Biosynthesis and utilization of structurally diverse norcobamide cofactors in the tetrachloroethene-respiring bacterium *Sulfurospirillum multivorans*

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List of abbreviations

Ado	5'-deoxyadenosyl-
AP-P	(*R*)-1-aminopropan-2-ol *O*-2-phosphate
α-RP	α-ribotide (N1-(5-phospho-alpha-d-ribosyl)-base)
Bza	benzimidazole
(Bza)	benzimidazole (general abbreviation for all kinds of benzimidazoles)
Cba	cobamide
Cbi(-P)	cobinamide (phosphate)
Cbi-GDP	cobinamide guanosine diphosphate
Cby	cobyric acid
*c*DCE	*cis*-1,2-dichloroethene
$(CN)_2$Cbi	dicyanocobinamide
$(CN)_2$Cby	dicyanocobyric acid
DMB	5,6-dimethylbenzimidazole
DNO_2Bza	5,6-dinitrobenzimidazole
DOMeBza	5,6-dimethoxybenzimidazole
EA-P	ethanolamine *O*-phosphate
L-Ser-P	L-serine *O*-phosphate
L-Thr-P	L-threonine *O*-3-phosphate
MeBza	methylbenzimidazole
NaMN	nicotinate mononucleotide
NCba	(176)-norcobamide
OHBza	hydroxybenzimidazole
OHRB	organohalide-respiring bacteria
OMeBza	methoxybenzimidazole
PCE	tetrachloroethene
PLP	pyridoxal-5'-phosphate
TCE	trichloroethene

Summary/ Zusammenfassung

The respiratory tetrachloroethene (PCE) reductive dehalogenase PceA of the ε-proteobacterium *Sulfurospirillum multivorans* harbors the unusual cobamide cofactor norpseudo-B_{12} that is *de novo* synthesized by this bacterium. Norpseudo-B_{12} contains an adenine as lower base and a unique ethanolamine *O*-phosphate (EA-P) as linker moiety in its nucleotide loop. Compared to PceA homologs that utilize cobamides with other bases, mainly benzimidazoles, and an (*R*)-1-aminopropan-2-ol *O*-2-phosphate (AP-P) linker with an additional methyl group compared to EA-P, *Sm*PceA displayed a drastically higher activity. The special nucleotide loop of norpseudo-B_{12} was previously shown to be involved in binding to PceA. The aims of this study were to investigate the biosynthesis of the linker and lower base of norpseudo-B_{12} and to analyze the variability of cobamides producible by *S. multivorans* plus their effects on the PCE-metabolism. The exogenously applied benzimidazoles (Bza) 5,6-dimethyl-Bza (DMB), 5-methyl-Bza (5-MeBza), Bza, 5-hydroxy-Bza (5-OHBza), and 5-methoxy-Bza (5-OMeBza) efficiently replaced adenine in norpseudo-B_{12}. The analysis of the lower ligand activating enzyme *Sm*CobT revealed an activation of the singly substituted 5-OMeBza, 5-OHBza, and 5-MeBza to a mixture of α-ribotides (α-RP) with the substituent at C5 and C6 of the benzimidazole with a predominant synthesis of the C6 isomer only in the case of 5-OHBza activation. Exclusively 5-OMeBza-, 6-OHBza- and both 5- and 6-MeBza norcobamide (NCba) were synthesized by *S. multivorans* revealing a specificity in the cobamide biosynthesis for certain CobT products. Most (Bza)-NCbas efficiently replaced norpseudo-B_{12} in PceA by capturing the same position in the active site, which had no effects on the PCE-metabolism. Only DMB-NCba showed strong negative effects on the PCE-metabolism of *S. multivorans* due to a highly inefficient incorporation of this cobamide into PceA. The key enzyme synthesizing the unique EA-P linker was analyzed as novel L-serine *O*-phosphate (L-Ser-P) decarboxylase *Sm*CobD. Besides L-Ser-P this enzyme additionally decarboxylated L-threonine *O*-3-phosphate (L-Thr-P) to AP-P *in vitro* with a lower conversion rate. At high exogenous concentrations of L-Thr-P *S. multivorans* predominantly synthesized the AP-P containing pseudo-B_{12}, which was poorly incorporated into *Sm*PceA affirming its specificity for NCba cofactors. This study unraveled the special biosynthesis of the unique norpseudo-B_{12} in *S. multivorans* and the exceptional variability of cobamides and norcobamides that can be produced by this organism. Moreover, new relevant insights into the binding of cobamide cofactors to target enzymes were achieved.

Zusammenfassung

Die respiratorische Perchlorethen (PCE) reduktive Dehalogenase PceA des ε-Proteobakteriums *Sulfurospirillum multivorans* bindet den einzigartigen Cobamid-Kofaktor Norpseudo-B_{12}. Dieses, von *S. multivorans de novo* biosynthetisierte, Cobamid, besitzt Adenin als untere Base und Ethanolamin-*O*-Phosphat (EA-P) als einzigartiges Linkermolekül in seinem Nukleotid-Loop. Andere PceA-Homologe nutzen Cobamide mit anderen unteren Basen, vor allem Benzimidazole, und (*R*)-1-Aminopropan-2-ol-*O*-2-Phosphat (AP-P) mit einer Methylgruppe mehr als EA-P als Linker. *Sm*PceA hat eine deutlich höhere Aktivität als andere PceA Enzyme. Die Strukturaufklärung von PceA zeigte, dass der Nukleotid-Loop von Norpseudo-B_{12} zur Bindung zum Protein beiträgt. Das Ziel dieses Projektes war die Untersuchung der Biosynthese der unteren Basen und des Linkers von Norpseudo-B_{12} und der Variabilität von Cobamid-Kofaktoren, welche von *S. multivorans* produziert werden können, sowie deren Effekte auf den PCE-Metabolismus. Die exogene Zugabe der Benzimidazole (Bza) 5,6-Dimethyl-Bza (DMB), 5-Methyl-Bza (5-MeBza), Bza, 5-Hydroxy-Bza (5-OHBza) und 5-Methoxy-Bza (5-OMeBza) zeigte den effektiven Austausch von Adenin in Norpseudo-B_{12} mit den Bzas. Die *in vitro* Analyse der Phosphoribosyltransferase *Sm*CobT zeigte die Aktivierung der einfach-substituierten Benzimidazole 5-OMeBza, 5-OHBza und 5-MeBza zu einem Mix aus α-Ribotiden (α-RPs) mit dem jeweiligen Liganden an C5 oder C6 des Benzimidazoles. Dennoch wurden nur 5-OMeBza, 6-OHBza sowie beide 5- und 6-MeBza α-RP von *S. multivorans* in Norcobamide (NCba) eingebaut, was ein neuer Beweis für die Spezifizität in der B_{12}-Biosynthese für gewisse CobT-Produkte ist. Einige (Bza)-NCba haben Norpseudo-B_{12} in PceA effektiv durch die Einnahme derselben Struktur im aktiven Zentrum ersetzt, was auch keinen Effekt auf den PCE-Metabolismus zeigte. Dieser wurde von DMB-NCba durch einen äußerst schlechten Einbau des Cobamides in PceA stark negativ beeinflusst. Die neuartige L-Serin-*O*-Phosphat-(L-Ser-P)-Decarboxylase *Sm*CobD wurde als das EA-P Linker-herstellende Enzym in *S. multivorans* bestimmt. CobD konnte auch L-Threonin-*O*-3-Phosphat (L-Thr-P) zu AP-P mit einer geringeren Rate decarboxylieren. Bei hohen Konzentrationen von exogenem L-Thr-P hat *S. multivorans* fast ausschließlich AP-P-haltiges Pseudo-B_{12} synthetisiert, welches schlecht in PceA aufgenommen wurde. Mit dieser Studie wurde die Biosynthese des einzigartigen Norpseudo-B_{12} und diverser Cobamide und Norcobamide in *S. multivorans* aufgedeckt. Wichtige neue Erkenntnisse über die Bindung der Cobamid-Kofaktoren in B_{12}-abhängigen Enzymen wurden ebenfalls gewonnen.

1 Introduction

1.1 Cobamide structure and B_{12}-dependent enzymes

Cobamides (derivatives of vitamin B_{12}) are the most complex non-polymeric natural compounds (Eschenmoser 1988, Kräutler *et al.* 1998). They function as essential cofactors of diverse enzymes in prokaryotes, lower eukaryotes, humans and animals (Buckel and Golding 2008). The B_{12}-dependent enzymes are traditionally grouped as coenzyme B_{12}-dependent enzymes, cobamide-dependent methyltransferases, and cobamide-harboring reductive dehalogenases (Marsh 1999, Gruber *et al.* 2011). These methyltransferases catalyze the specific transfer of methyl groups and reductive dehalogenases abstract halogen atoms from organohalides under reductive conditions (Wohlfarth and Diekert 1999). Different coenzyme B_{12}-dependent enzymes perform deaminations, dehydrations, carbon skeleton rearrangements, or the reduction of ribonucleotides (Marsh 1999, Gruber *et al.* 2011). Recent investigations established the new class of cobamide-dependent S-adenosylmethionine enzymes that perform radical rearrangements or methylations (Bridwell-Rabb and Drennan 2017). The cobamide-dependent enzymes are involved in several essential ana- and catabolic pathways. For example in mammals the catabolism of amino acids, cholesterol, and odd-chain fatty acids involves the function of cobamide enzymes (Banerjee and Chowdhury 1999, Rosenblatt and Fenton 1999, Banerjee and Vlasie 2002). Thus, cobamides with their complex and unique structure are indispensable molecules in a vast number of organisms. These macrocyclic compounds, which are exclusively synthesized by a subset of prokaryotes (see 1.2), consist of an invariable core fused to variable sub-structures (Kratky and Kräutler 1999, Randaccio *et al.* 2006). The core structure is a conserved tetrapyrrolic ring system coordinating a central cobalt ion, the so-called corrin ring (see Fig. 1.1). This cobalt ion is tetra-coordinatively bound by the nitrogen atoms of the surrounding pyrrole rings. All molecules containing a corrin ring are termed corrinoids (IUPAC 1975). In cobamides (complete corrinoids) the cobalt ion is additionally coordinated by one or two variable axial ligands. The upper or β-ligand in coenzyme B_{12} derivatives is usually a 5'-deoxyadenosyl group (Fig. 1.1) that is bound to cobalt via a unique organometallic cobalt carbon (Co-C) bond (Warren *et al.* 2002). Other physiologically relevant upper ligands are a methyl group, or in reductive dehalogenases a water/hydroxyl moiety (Friedrich 1975, Bommer *et al.* 2014). When cobamides are extracted from natural sources the physiological β-ligand is typically replaced

by a cyano group that is derived from potassium cyanide used for purification (Veer *et al.* 1950, Wijmenga *et al.* 1950, Spalla *et al.* 1989). Hence, the cobamides get a unique upper cyano ligand and become stable at room temperature, since the Co-C bond in adenosylated and methylated cobamides is very sensitive to temperature and light (Kräutler 1998). The lower ligand is the terminal end of the so-called nucleotide loop, which is connected to the pyrrole ring D of the corrin ring (Kratky and Kräutler 1999, Randaccio *et al.* 2006). A part of this loop is the linker structure that is derived from (*R*)-1-aminopropan-2-ol *O*-2-phosphate (AP-P) in almost all known natural occurring cobamides (Lenhert 1968). Exclusively norpseudo-B_{12} (Co_{α}-adeninyl-176-norcobamide), the cobamide produced by the Gram-negative ε-proteobacteria *Sulfurospirillum multivorans* and *Sulfurospirillum halorespirans*, possesses an ethanolamine *O*-phosphate (EA-P) linker lacking a methyl group compared to AP-P (Scholz-Muramatsu 1995, Kräutler *et al.* 2003, Goris *et al.* 2017).

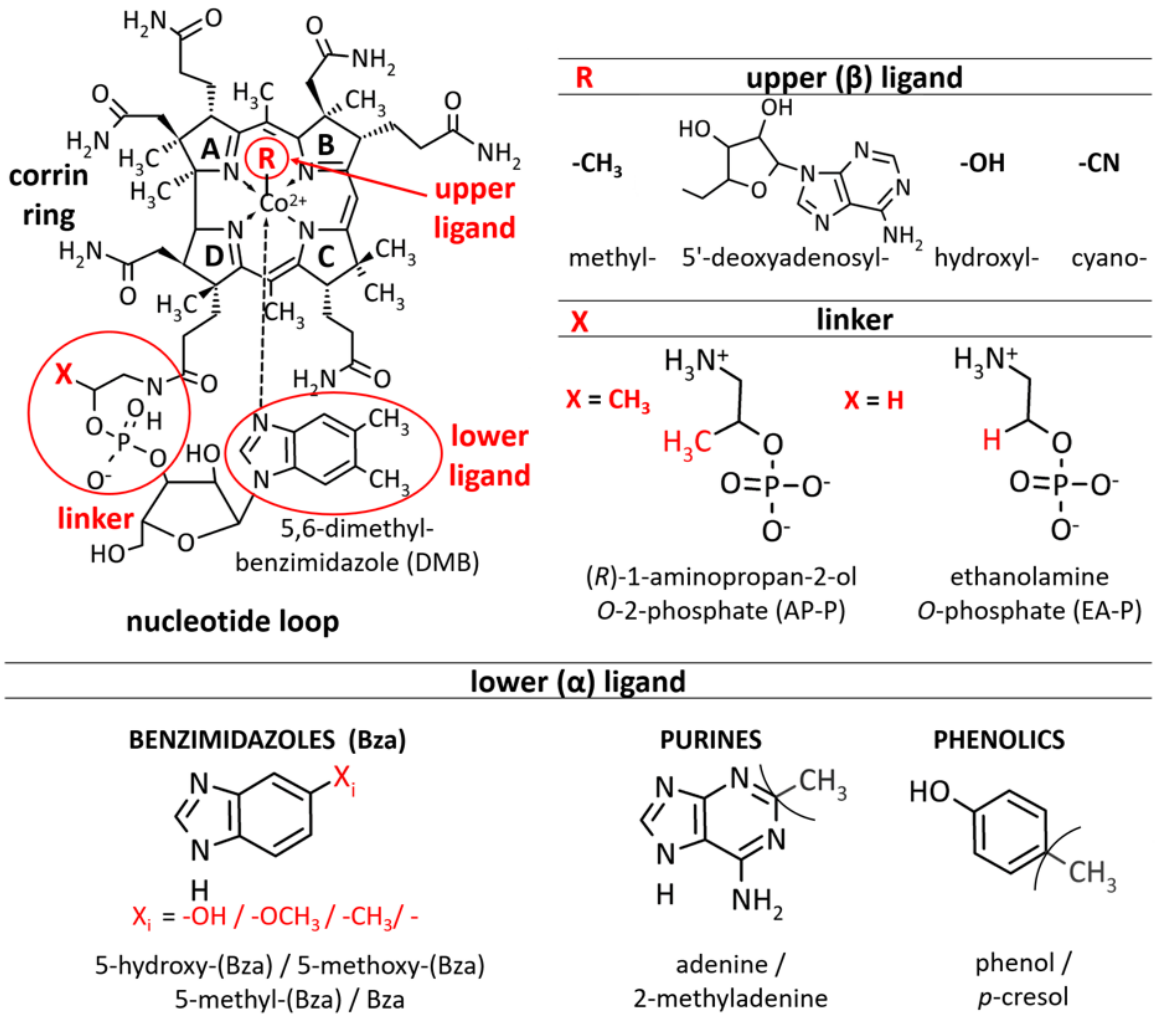

Figure 1.1: Cobamide structure with conserved and variable sub-structures (*red*).

The lower α-ligand is the most variable sub-structure and so far more than 16 different purines, benzimidazoles, or phenolic compounds (Fig. 1.1) have been found at this position in natural cobamides (Renz 1999, Schubert T. unpublished data). Phenol and *p*-cresol are the only known phenolic compounds at this position and were exclusively detected in members of the bacterial genera *Sporomusa* and *Veillonella* so far (Stupperich *et al.* 1989 a, b, Crofts *et al.* 2013). Benzimidazoles like 5,6-dimethylbenzimidazole (DMB) or 5-hydroxybenzimidzole (5-OHBza) and purines, predominantly adenine or 2-methyladenine, are the common α-ligands of B_{12}-derivatives and found among all cobamide synthesizing genera (Renz 1999, Taga and Walker 2008). The cobalt coordinating fraction of the lower ligand is a nitrogen atom of the imidazole ring. Thus, only purine and benzimidazole analogs can coordinate the cobalt ion and phenolics always stay de-coordinated from it, a state termed 'base-off' conformation (Stupperich 1990, Renz 1999, Gruber *et al.* 2011). The inability of the phenolics to coordinate the cobalt ion proofs the designation lower ligand incorrect for these molecules. However, due to the fact that they replace purines or benzimidazoles in some cobamides all three types are typically summarized as lower ligands (Cheong *et al*. 2001). Benzimidazoles and purines are switched between the cobalt coordinating 'base-on' and the 'base-off' conformation by protonation of the respective nitrogen atom depending on the cobamide containing enzyme. In cobamide-dependent methyltransferases the lower ligand is typically in 'base-off' conformation and the coordination to the cobalt center is taken over by a histidine of the protein backbone that belongs to the B_{12} binding motif DxHxxG (Drennan *et al.* 1994, Gruber *et al.* 2011). This conformation is termed 'base-off/His-on'. Cobamides with a methyl group as upper ligand like methylcob(III)alamin (Co_{α}-5,6-dimethylbenzimidazolyl-Co_{β}-methyl-cobamide) and their super-reduced Co^{+} state (e.g. cob(I)alamin) with both, a detached histidine and a de-coordinated lower ligand ('base-off/His-off') are the cofactors of these enzymes. The super-reduced cobamide abstracts a methyl group from typically N^5-methyltetrahydrofolate yielding the oxidized (Co^{3+}), hexa-coordinated methylcob(III)amide with 'base-off/His-on' conformation. By transfer of this methyl group to the substrate and a simultaneously detachment of the histidine from the cobalt ion due to a protonation of this amino acid the super-reduced cob(I)amide is restored. Coenzyme B_{12} dependent enzymes use Co_{β}-adenosylated cobamides like coenzyme B_{12} (Co_{α}-5,6-dimethylbenzimidazolyl-Co_{β}-5'-deoxyadenosyl cobamide) and catalyze a broad range of reactions based on 1,2-isomerizations of protein bound radicals (Gruber *et al.* 2011). The key process is the reversible

homolytic cleavage of the organometallic Co-C bond between the cobalt ion and the adenosyl group yielding a cob(II)amide and a 5'-deoxyadenosyl radical. This highly active nucleophilic radical creates a substrate radical by abstracting a hydrogen atom from the substrate. After a rapid 1,2-rearrangement within this structure a product radical is formed that re-abstracts the hydrogen atom from 5'-deoxyadenosine. Finally the 5'-deoxyadenosyl radical re-connects with cob(II)alamin to regenerate the adenosyl cob(III)amide. Different coenzyme B_{12}-dependent enzymes were revealed binding the cobamide in 'base-on' (e.g. diol dehydratases, ethanolamine ammonia lyase) or in 'base-off/His-on' conformation (e.g. methylmalonyl-CoA mutase, glutamate mutase) (summarized in Gruber *et al.* 2011). The B_{12}-dependent reductive dehalogenases (RDases) are exclusively found in a subset of anaerobic or microaerophilic bacterial species (Fetzner 1998, Fincker and Spormann 2017). Compared to coenzyme B_{12}-dependent enzymes and cobamide methyltransferases, whose reaction mechanisms are well understood, less is known about RDases (Wohlfarth and Diekert 1999, Bommer *et al.* 2014). With help of the cobamide in its super-reduced Co(I)-'base-off' state these enzymes abstract halogen atoms, typically chlorine or bromine, from organohalides and replace them with protons under anaerobic conditions (Fetzner 1998). Structural characterization of two reductive dehalogenases (PceA from *Sulfurospirillum multivorans* and *Np*RdhA from *Nitratireductor pacificus* pht-3B) revealed that the cobamide cofactor shifts between the Co(I)-'base-off' and the Co(II)-'base-off' state with a hydroxyl or water group as upper ligand of the latter (Bommer *et al.* 2014, Payne *et al.* 2015). Unlike other 'base-off' enzymes, RDases lack the histidine for the 'His-on' conformation and hence their catalysis involves no lower ligand binding to the cobalt ion. This 'base-off/His-off' conformation was also found in several cobamide-dependent S-adenosylmethionine radical enzymes (Bridwell-Rabb and Drennan 2017). The electron reducing Co(II) to Co(I) of the cobamide cofactor in RDases is delivered by a proximal iron-sulfur cluster in both investigated enzymes. However, the electron transfer mechanism from the cobamide to the substrate seems to be different. While in *Np*RdhA a substrate halogen covalently binds the cobalt ion, the substrate showed no binding to the cobalt in PceA. Thus, reductive dehalogenases might offer diverse reaction mechanisms. Typical organohalides are halogenated alkanes or alkenes (e.g. tetrachloroethene (PCE), tribromoethane (TBA)), benzenes, biphenyls, and dioxins (Fetzner 1998). They represent abundant pollutants of the environment due to natural and mostly anthropogenic influences (Sutfin 1996). Diverse bacteria couple the reductive dehalogenation to energy conservation, a

process called organohalide respiration (Holliger *et al.* 1998, Fetzner 1998, Hug *et al.* 2013, Fincker and Spormann 2017). Organohalide respiring bacteria (OHRB) were found to belong to the genera γ-, δ-, ε-Proteobacteria, Firmicutes, and Chloroflexi. Besides some OHRB whose metabolism is restricted to organohalide respiration (e.g. *Dehalococcoides mccartyi*, *Dehalobacter* spp.) other bacteria show a versatile metabolism (e.g. *Desulfitobacterium hafniense*, *Sulfurospirillum multivorans*).

1.2 Cobamide *de novo* biosynthesis

The *de novo* biosynthesis of cobamides is exclusively performed by some prokaryotic species in a more than 25 enzymes involving pathway (Renz 1999, Warren 2002). Two pathways for cobamide biosynthesis are known depending on the absence or presence of oxygen. In the oxygen-dependent pathway the insertion of cobalt during corrin ring assembly is performed at a later step compared to the anaerobic route. The precursor for cobamide biosynthesis is uroporphyrinogen III, which also serves as progenitor of other tetrapyrroles like heme, siroheme, or chlorophyll (Battersby 2000). Uroporphyrinogen III originates from L-glutamate in most prokaryotes excluding α-proteobacteria (Battersby and Leeper 1999). The latter produce it via direct combination of L-glycine and succinyl CoA (Shemin pathway). A scheme for the anaerobic coenzyme B_{12} biosynthesis in Gram-negative bacteria is depicted in Figure 1.2. The conversion of uroporphyrinogen III to the corrin ring compound adenosylcobyric acid (AdoCby) involves methylations, amidations, the insertion and adenosylation of cobalt, and a ring contraction resulting in a direct linkage between the pyrrole rings A and D (Fig. 1.1). About 15 enzymes are necessary for these steps. Even though a lot of enzymes are necessary for the corrin ring formation, the cobamide core is synthesized structurally identical in all known *de novo* synthesizing prokaryotes. The addition of the 5'-deoxyadenosyl (Ado) group to the cobalt ion is catalyzed by so called ATP:cob(I)amide adenosyltransferases (EC 2.5.1.17) that synthesize Ado by an unusual hydrolysis of ATP producing triphosphate as side product (Fonseca and Escalante-Semerena 2001, Fonseca *et al.* 2002). So far three different types of enzymes catalyzing this reaction, namely CobA, EutT, and PduO, are known (Mera and Escalante-Semerena 2010). The next step after the assembly of AdoCby is the biosynthesis and incorporation of the AP-P linker (Warren *et al.* 2002). This pathway was first described for the γ-proteobacterium *Salmonella enterica* serovar *thyphimurium* LT2 that has a complete set

of genes for the anaerobic cobamide biosynthesis pathway (Jeter *et al.* 1984, Brushaber *et al.* 1998). The first step in AP-P biosynthesis is catalyzed by the L-threonine kinase PduX (EC 2.7.1.177) that phosphorylates the amino acid L-threonine to L-threonine *O*-3-phosphate (L-Thr-P) by use of ATP (Fan and Bobik 2008a). A *S. enterica pduX* deletion strain showed a slow growth under conditions that require the biosynthesis of coenzyme B_{12}.

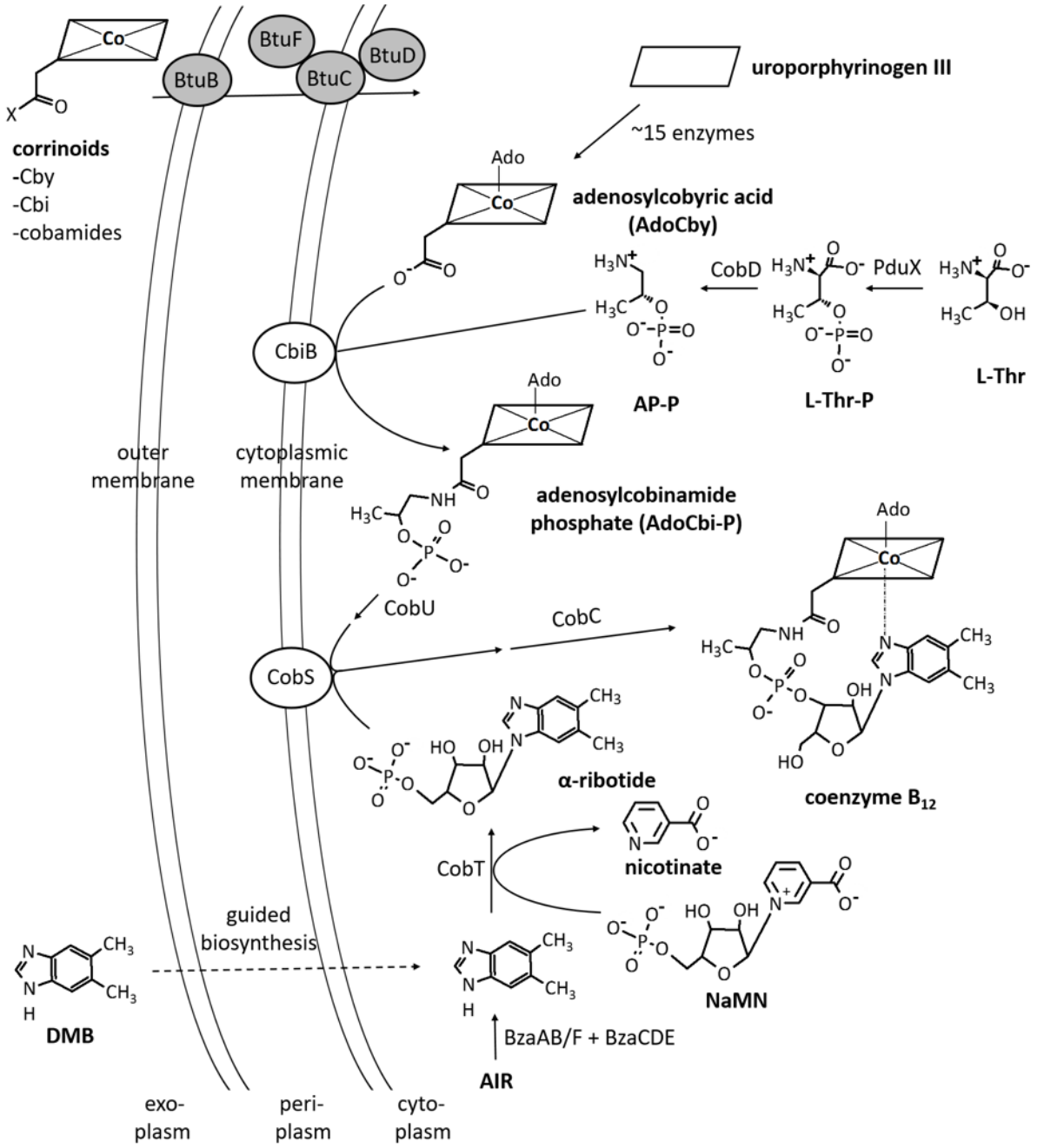

Figure 1.2: Anaerobic biosynthesis of coenzyme B_{12} in Gram-negative bacteria. The conserved corrin ring is represented as rectangle with a tetra-coordinated cobalt ion. Details of the cell wall are omitted for a better clarity. AIR, 5'-aminoimidazole ribotide.

This finding revealed the important role of this enzyme for the *de novo* biosynthesis of cobamides and the slow growth suggested a second unknown L-threonine kinase with a low activity. The PduX product L-Thr-P is subsequently decarboxylated to AP-P by the L-threonine *O*-3-phosphate decarboxylase CobD (EC 4.1.1.81, Brushaber *et al.* 1998). This enzyme removes the carboxyl group from L-Thr-P with help of the prosthetic pyridoxal-5'-phosphate (PLP) cofactor (John 1995, Percudani and Peracchi 2013). The third and final step in the biosynthesis of the AP-P linker is its addition to pyrrole moiety D of the corrin ring (AdoCby). This ATP dependent reaction is catalyzed by the adenosylcobinamide phosphate synthase CbiB (EC 6.3.1.10), which is a membrane protein (Zayas *et al.* 2007). Both enzymes, CobD and CbiB, were shown to be essential for the cobamide biosynthesis in *S. enterica*. The *cobD* or *cbiB* deletion strains displayed no growth under cobamide-depending conditions unless the missing gene was heterologously introduced into the bacterium or the enzyme products were added to the cultivation media (Brushaber *et al.* 1998, Zayas *et al.* 2007). Interestingly, when EA-P was added to the medium the cobamides produced in *S. enterica* Δ*cobD* were norcobamides with an EA-P rather than an AP-P linker (Fig. 1.1). This finding revealed that CbiB of *S. enterica* can attach both AP-P and EA-P to the corrin ring. The final step of the cobamide biosynthesis is the four enzymes involving nucleotide loop assembly pathway (Maggio-Hall and Escalante-Semerena 1999, Escalante-Semerena 2007). The bifunctional adenosylcobinamide kinase/ adenosylcobinamide-phosphate guanylyltransferase CobU (EC 2.7.1.156 and 2.7.7.62) converts the CbiB product AdoCbi-P to AdoCbi-GDP (adenosylcobinamide guanosine diphosphate) by addition of GMP derived from the cleavage of GTP (Blanche *et al.* 1991, Thomas *et al.* 2000). The second function of this enzyme, the phosphorylation of adenosylcobinamide, is not required during *de novo* B_{12}-biosynthesis, but for the salvaging of exogenous non-phosphorylated cobinamide (O'Toole and Escalante-Semerena 1993). The lower ligand is activated by so called nicotinate mononucleotide:5,6-dimethylbenzimidazole phosphoribosyltransferases (EC 2.4.2.21, Trzebiatowski *et al.* 1994). Most B_{12}-synthesizers contain a homodimeric CobT able to activate certain benzimidazoles and purines, but not phenolics (Crofts *et al.* 2013). Some bacteria have instead a heterodimeric ArsAB complex activating especially phenolics, but also benzimidazoles (Chan and Escalante-Semerena 2011). The activation of lower ligands is the replacement of nicotinate from β-nicotinate mono nucleotide (NaMN) with the base resulting in α-ribotides with the lower ligand bound to C1 of ribose in α-orientation (Fig. 1.2). It was also shown that CobT homologs

are able to use NAD^+ (nicotinamide adenine dinucleotide) instead of NaMN and *in vitro* assays additionally revealed functional enzymes with nicotinamide mono nucleotide or nicotinate adenine dinucleotide (Maggio-Hall and Escalante-Semerena 2003, Hazra *et al.* 2013). However, by far the best activity of the enzymes were obtained with NaMN. The activated lower ligand (α-ribotide) and AdoCbi-GDP are subsequently combined by the membrane protein CobS (adenosylcobalamin-5'-phosphate synthase, EC 2.7.8.26, Maggio-Hall *et al.* 2004). The final step yielding coenzyme B_{12} is the removal of the phosphate group at C5 of the ribose moiety by the phosphatase CobC (EC 3.1.3.73, O'Toole *et al.* 2004, Zayas and Escalante-Semerena 2007). Many prokaryotes are able to salvage exogenous corrinoids (e.g. Cby, Cbi, or cobamides) by a specific transporter complex composed of the proteins BtuCDF (EC 3.6.3.33, Locher and Borths 2004). This ABC (ATP Binding Cassette) transporter complex is located at the cytoplasmic membrane and speficically transfers corrinoids into the cytoplasm. However, Gram-negative bacteria additionally require the outer membrane TonB dependent transporter BtuB (EC 3.6.3.33) for the specific import of exoplasmic corrinoids into the periplasm (DiMasi *et al.* 1973, Kadner and Liggins 1973). The background of the variability in the lower ligand structure of cobamides was unraveled by an investigation of the CobT and ArsAB homologs of different prokaryotes (Crofts *et al.* 2013, Hazra *et al.* 2013). Most CobT and ArsAB enzymes showed a broad substrate range towards different lower base precursors *in vitro* and *in vivo*. These enzymes are typically not just able to activate one specific lower ligand, but several ones with different specificities. Additionally it was shown that these phosphoribosyl transferases also activate lower ligand precursors that are taken up by the cell from the environment, a process termed guided biosynthesis (Renz 1999). For example CobT of *S. enterica* can activate adenine and 2-methyladenine, the native cobamide lower ligands in this bacterium under anaerobic growth conditions (Anderson *et al.* 2008), but also several benzimidazoles (Crofts *et al.* 2013). The CobT homolog of the chloroflexi *Dehalococcoides mccartyi* strain 195 is able to activate certain benzimidazoles, but not purines. Furthermore this strictly OHRB, which lacks the genes for the corrin ring biosynthesis and thus requires Cby, Cbi, or a cobamide from the environment, can grow efficiently on trichloroethene (TCE) only with cobamides bearing DMB, 5-methylbenzimidazole (5-MeBza), or 5-methoxybenzimidazole (5-OMeBza) as lower ligand (Yi *et al.* 2012). The *D. mccartyi* strains GT and BAV1 were investigated to require cobamides with the same lower ligands like strain 195 or benzimidazole for their *cis*-1,2-dichloroethene (*c*DCE)-to-ethene dehalogenating RDases VcrA

or BvcA, respectively, when grown with *c*DCE (Yan *et al.* 2013). However, when strain GT was grown with vinyl chloride only DMB and 5-MeBza-harboring cobamides allowed the bacterium to grow. The PceA containing OHRB *Desulfitobacterium hafniense* strains Y51 and DCB-2 containing a complete set of genes for the anaerobic biosynthesis of a cobamide with purine as lower ligand (Co_{α}-purinyl-Co_{β}-5'-deoxyadenosyl-cobamide) were shown to grow equally on PCE medium amended with different types of benzimidazoles and purines (Reinhold *et al.* 2012, Schubert T. unpublished data). Moreover, all tested exogenous benzimidazoles effectively replaced purine as cobamide lower ligand in these bacteria. These results revealed that different OHRB possess CobT homologs with a restricted or wide substrate range as well as RDases with specific or unspecific requirements for the lower ligand of the cobamide cofactor even depending on the organohalide substrate. In case of guided biosynthesis with benzimidazoles containing one ligand at the benzene ring (i.e. 5-OMeBza and 5-OHBza) some CobT and ArsAB proteins were shown to produce two different α-ribotides, one with the substituent at C5 and one at C6 (Crofts *et al.* 2014). For example *S. enterica* CobT produced almost exclusively 5-OMeBza α-ribotide from 5-OMeBza and similar amounts of 5- and 6-OHBza α-ribotide from 5-OHBza. The endogenous source of free purine and phenol derivatives for lower ligand activation was suggested to be the degradation of tRNA for purines and amino acids like L-tyrosine for the phenolics (Renz 1999). Benzimidazoles are synthesized by certain enzymes that usually are clustered within the cobamide biosynthesis genes. Under aerobic conditions DMB is synthesized by the DMB synthase BluB (EC 1.14.99.40, Taga *et al.* 2007). This enzyme cleaves reduced flavin mononucleotide to DMB and D-erythrose 4-phosphate. In anaerobic bacteria benzimidazoles are formed by the gene products of *bzaABCDEF* (Hazra *et al.* 2015). The precursor for this pathway is AIR (5-aminoimidazole ribotide) that is a central intermediate in the formation of purines and thiamine (Chatterjee *et al.* 2001). The gene products of *bzaAB* or *bzaF* (EC 4.1.99.M1) convert AIR to 5-OHBza, which is methylated to 5-OMeBza by BzaC (EC 2.1.1.M9). BzaD (EC 2.1.1.M10) further methylates 5-OMeBza to 5-methoxy-6-methylbenzimdazole. Finally DMB is formed by the activity of BzaE. The presence of *bzaABCDE* or *bzaFCDE* in the genome of prokaryotes implies the synthesis of DMB as lower ligand and the lack of certain genes the respective use of a DMB precursor. Most prokaryotes lacking the genes for the benzimidazole biosynthesis use adenine or more rarely other purines or phenolics as cobamide lower ligand (Taga and Walker 2008).

1.3 PCE metabolism and norpseudo-B_{12} biosynthesis in *S. multivorans*

The Gram-negative organohalide-respiring ε-proteobacterium *S. multivorans* possesses a respiratory tetrachloroethene reductive dehalogenase PceA (EC 1.97.1.8) that dechlorinates PCE to *c*DCE via TCE (Neumann *et al.* 1994, Scholz-Muramatsu *et al.* 1995). This RDase is located at the periplasmic face of the cytoplasmic membrane and is most probably anchored to it by the small hydrophobic protein PceB (Neumann *et al.* 1998). Besides PCE and TCE this organism can grow with other electron acceptors like fumarate, nitrate, or even with up to 5 % atmospheric oxygen (Scholz-Muramatsu *et al.* 1995, Goris *et al.* 2014). Typical electron donors are hydrogen, formate, and pyruvate. The unusual cobamide cofactor of PceA in *S. multivorans* is norpseudo-B_{12} with an adenine as lower ligand and the unique EA-P linker in its nucleotide loop (Fig. 1.3A, Kräutler *et al.* 2003). Besides this cobamide PceA contains two [4Fe-4S]-clusters. During assembly of the protein the cofactors are incorporated into a cytoplasmic precursor of PceA bearing a twin arginine translocation signal for transport across the cytoplasmic membrane (John *et al.* 2006, Palmer and Berks 2012). Compared to PceA homologs from other bacteria the *S. multivorans* enzyme revealed an at least four times higher specific activity with 2640 nkat/mg protein (Schubert and Diekert 2016). Purified PceA enzymes from *Desufitobacterium hafnienense* PCE-S or *Dehalococcoides mccartyi* 195 harbored cobamides with an AP-P linker and purine or DMB as lower ligand and displayed specific activities of 650 or 342 nkat/mg, respectively. Cobamides are also able to dechlorinate abiotically (Neumann *et al.* 2002). Isolated norpseudovitamin B_{12} (Co_{α}-adeninyl-Co_{β}-cyano-176-norcobamide) from *S. multivorans* revealed a three times higher dechlorination activity of trichloroacetate than pseudovitamin B_{12} at pH 7.5 (717 vs. 250 mol Cl^- released per mol cobamide per second. The latter differs from norpseudovitamin B_{12} only in the AP-P linker with an additional methyl group (Taga and Walker 2008). Compared to vitamin B_{12} with DMB as lower ligand and an AP-P linker (14 s^{-1}) the *S. multivorans* cobamide displayed even a 50-fold higher activity (Neumann *et al.* 2012). Both, the EA-P linker and the lower ligand adenine seemed to enhance the reductive dechlorination capability of free and enzyme-bound norpseudo-B_{12} compared to other cobamides. However, it was not known why *S. multivorans* exclusively synthesizes the exceptional EA-P containing norcobamide and not an AP-P cobamide like other prokaryotes. In the PceA structure the carbon atom C176 of the EA-P moiety of norpseudo-B_{12} is in such close proximity to a β-sheet of the enzyme that the methyl group at C176 as found in AP-P cobamides seemed not fit into this position (Bommer *et al.*

2014). Hence, a strict requirement of PceA for norcobamides was assumed. Nevertheless, no evidence for this hypothesis was available. *S. multivorans* contains a complete set of genes for the anaerobic biosynthesis of norpseudo-B_{12} (Fig. 1.3B). These genes are clustered together in one operon directly downstream of the RDase gene cluster including *pceA* and its putative membrane anchor *pceB* (Goris *et al.* 2014). The cobamide biosynthesis genes of *S. multivorans* are most similar to the homologs from the non-dehalogenating *Ilyobacter polytropus.* Interestingly, the genome of *S. multivorans* lacks a gene encoding an ATP:cob(I)amide adenosyltransferase responsible for the adenosylation of the cobalt ion during corrin ring biosynthesis. Most probably the cobalt center of norpseudo-B_{12} is not adenosylated during cobamide biosynthesis.

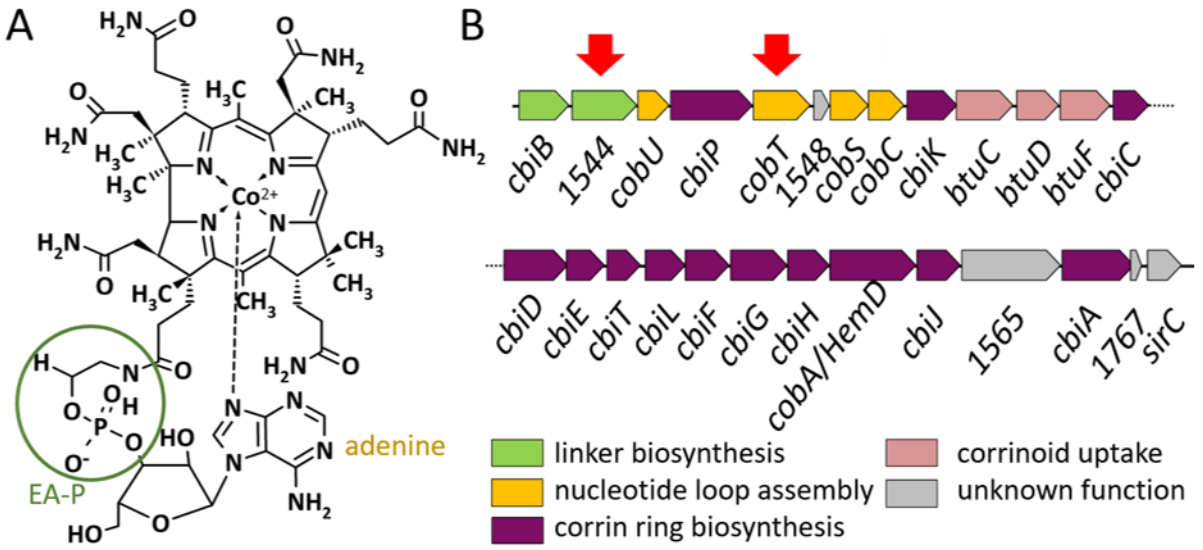

Figure 1.3: Structure (A) and biosynthesis gene cluster (B) of norpseudo-B_{12} in *S. multivorans* (B). The red arrows indicate the predicted *cobD* homolog *SMUL_1544* and *cobT*.

The first two genes of the B_{12} biosynthesis gene cluster in *S. multivorans* were assumed to encode the linker biosynthesis proteins CbiB and a putative CobD homolog (SMUL_1544) that shares about 22 % sequence identity to the characterized and 28 amino acids shorter L-Thr-P decarboxylase CobD of *S. enterica* (Brushaber *et al.* 1998). These enzymes were hyphotized to be responsible for the formation and incorporation of the unique EA-P linker into norpseudo-B_{12}, but no evidence was available verifying this hypothesis. Compared to *S. enterica* and other cobamide synthesizers a gene encoding a PduX-like L-threonine kinase is missing in *S. multivorans*. Hence it was assumed that the amino acid L-threonine did not serve as progenitor of the EA-P cobamide linker in the bacterium. Since norpseudo-B_{12} lacks the methyl

group at position C176 that is present in AP-P containing cobamides, an analogous linker pathway with L-serine instead of L-threonine as precursor was assumed for *S. multivorans*. This hypothesis included L-serine *O*-phosphate (L-Ser-P), which was assumed to be either generated by the activity of a so far unknown kinase or directly derived from the serine biosynthesis, where it serves as intermediate (Pizer 1963, Kräutler *et al.* 2003). Moreover, it was suggested that L-Ser-P is decarboxylated by SMUL_1544 yielding EA-P, which is subsequently attached to the corrin ring by CbiB. The lower ligand of the *S. multivorans* cobamide is an adenine molecule (Fig. 1.3A). In the active site of PceA this adenine is de-coordinated from the cobalt ion of the corrin ring (Bommer *et al.* 2014). Moreover the 'base-off' conformation of norpseudo-B_{12} in PceA is structurally unique due to a curled structure of the nucleotide loop including several hydrogen bonds between the adenine, the ribose moiety, the amide part of the EA-P linker and the carboxamide side chain at the C-ring of the corrin core. *S. multivorans* was the first OHRB investigated for its cobamide cofactor (Kräutler *et al.* 2003). The importance of adenine as lower ligand of norpseudo-B_{12} was hypothized by the tremendously higher abiotic dechlorination rates of trichloroacetate compared to DMB containing vitamin B_{12} (Neumann *et al*. 2002). Nevertheless, it was not known, if the *S. multivorans* reductive dehalogenase PceA strictly relies on the adenine containing norcobamide, or if it was also active with benzimidazole containing cofactors. The putative lower ligand activating enzyme CobT of this organism is encoded in the norpseudo-B_{12} biosynthesis gene cluster (Fig. 1.3B, *red arrow*). The ability of *S. multivorans* (*Sm*) to import exogenous benzimidazoles and incorporate them into norcobamides including the ability of *Sm*CobT to activate these bases was not known.

1.4 Aims of this study

An aim of this study was to unravel the unique EA-P cobamide linker biosynthesis pathway in *S. multivorans* and to investigate the dependency of PceA on norcobamide cofactors. The second aim was to analyze, if the lower base adenine of norpseudo-B_{12} could be replaced by exogenous benzimidazoles and if such novel norcobamides caused negative effects on PceA and the PCE metabolism of the organism. This aim involved the *in vitro* characterization of the substrate range of the lower ligand activation protein *Sm*CobT. The tested benzimidazole derivatives were DMB, 5-MeBza, Bza, 5-OHBza, and 5-OMeBza (Fig. 1.1).

2 Results

2.1 Exogenous 5,6-dimethylbenzimidazole caused production of a non-functional tetrachloroethene reductive dehalogenase in *Sulfurospirillum multivorans*

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Status of the manuscript: published

In this report an efficient uptake and incorporation of exogenous 5,6-dimethylbenzimiazole (DMB) as cobamide lower ligand in *S. multivorans* was revealed. The predominant synthesis of the novel cobamide cofactor nor-B_{12} (Co_{α}-5,6-dimethylbenzimidazolyl-176-norcobamide) showed drastic negative effects on the PCE-dependent growth and on the processing and activity of PceA due to a putatively inefficient incorporation of nor-B_{12} into the enzyme. Isolated DMB-containing norvitamin B_{12} revealed tremendously lower abiotic dechlorination activities of trichloroacetate than norpseudovitamin B_{12} harboring adenine as lower ligand.

Contribution of Sebastian Keller to this study: 70 %

Sebastian Keller performed the growth experiments of *S. multivorans*, the determination of the PceA activity and processing in crude extracts of the bacterium, the cobamide extraction and HPLC analysis in DMB-treated cells (Fig. 2-5). He performed the abiotic dechlorination assay with different isolated cobamides (Fig. 7) and he prepared the norvitamin B_{12} sample for the mass spectrometty (MS) and nuclear magnetic resonance (NMR) analysis.

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Exogenous 5,6-dimethylbenzimidazole caused production of a non-functional tetrachloroethene reductive dehalogenase in *Sulfurospirillum multivorans*

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Summary

Corrinoid-dependent reductive dehalogenation is mediated by phylogenetically diverse anaerobic bacteria that either synthesize corrinoids *de novo* or are dependent on corrinoid salvaging from the environment. The tetrachloroethene (PCE) reductive dehalogenase (PceA) of the Gram-negative Epsilonproteobacterium *Sulfurospirillum multivorans* harbours a norpseudo-B_{12} as corrinoid cofactor. Norpseudo-B_{12} differs from coenzyme B_{12} in the nucleotide loop structure. Adenine instead of 5,6-dimethylbenzimidazole (DMB) serves as lower ligand base of the central cobalt ion, and the nucleotide loop of norpseudo-B_{12} lacks a methyl group at position 176. In this study, *S. multivorans* was grown anaerobically with PCE in the presence of DMB. At a DMB concentration of 25 μM, the adenine moiety in the nucleotide loop of norpseudo-B_{12} was quantitatively replaced by DMB. The formation of the DMB-containing nor-B_{12} severely affected PCE-dependent growth and the PceA activity. In DMB-treated cells processing of the cytoplasmic PceA precursor was impeded, a result pointing to retarded cofactor incorporation. PceA enriched from cells cultivated with DMB contained nor-B_{12}. Nor-B_{12} purified from cells grown in the presence of DMB mediated the abiotic reductive dehalogenation of trichloroacetate to dichloroacetate at a 25-fold lower rate in comparison with norpseudo-B_{12}, a fact underpinning the relevance of norpseudo-B_{12} as efficient catalyst for reductive dehalogenation in general.

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Introduction

The dehalogenation of alkyl halides is mediated by reduced corrinoids either abiotically in a protein-released state (Glod *et al.*, 1997; Neumann *et al.*, 2002) or enzymatically, when bound as essential cofactors to reductive dehalogenases in anaerobic bacteria (Holliger *et al.*, 2003). These enzymes play a key role in organohalide respiration, i.e. the reductive dechlorination coupled to energy conservation via a chemiosmotic mechanism. Corrinoids are natural products exclusively synthesized in prokaryotes and indispensable for the function of a variety of enzymes, e.g. corrinoid-dependent methyltransferases, mutases, eliminases and reductive dehalogenases (reviewed in Banerjee and Ragsdale, 2003).

The complicated corrinoid structure is composed of a contracted tetrapyrrole macrocycle coordinating a cobalt ion in the centre (Lenhert and Hodgkin, 1961). In 'complete' corrinoids (henceforth referred to as cobamides), the cobalt centre is additionally coordinated by two axial ligands (see also Fig. 1A). The 'lower' ligand is part of an extensive nucleotide loop with a terminal base coordinating the cobalt ion at the α-side of the corrin ring (Warren *et al.*, 2002). The 'upper' ligand incorporated at the β-side during cobamide biosynthesis is a 5'-deoxyadenosyl moiety bound to the cobalt ion via a Co-C σ-bond. Natural cobamides were found to contain a variety of lower ligand bases (Renz, 1999). Besides the frequently found 5,6-dimethylbenzimidazole (DMB) (Fig. 1B), other benzimidazole or purine analogues were described. Especially adenine seems to play a common role as lower ligand base incorporated into cobamides when DMB is not available (Anderson *et al.*, 2008; Taga and Walker, 2008). So far, little is known about the structural diversity of cobamide cofactors of reductive dehalogenases and the variety of their lower ligand bases. The heterogeneous group of reductively dehalogenating bacteria can be divided into organisms able to synthesize corrinoids *de*

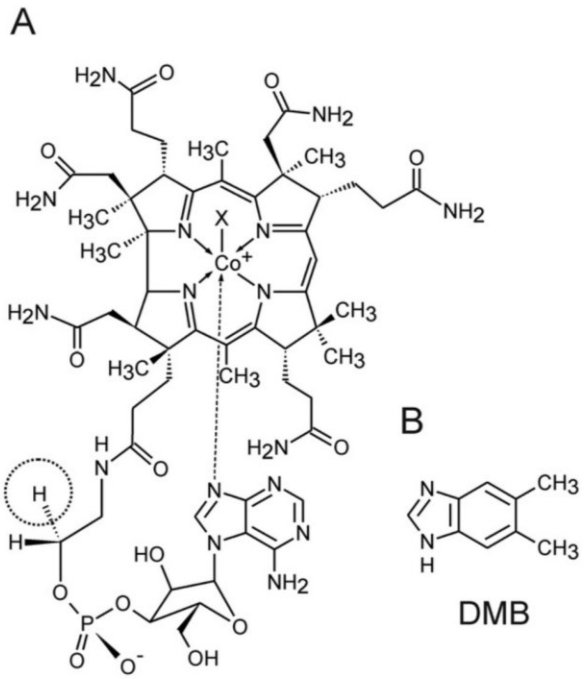

Fig. 1. Structure of the norpseudo-B_{12} cofactor of the tetrachloroethene (PCE) reductive dehalogenase (PceA) from *S. multivorans* in the *base-on* form with an adeninyl-moiety as lower ligand base (A) and of the alternative base 5,6-dimethylbenzimidazole (B). The bound hydrogen atom at position 176, which is replaced by a methyl group in pseudo-B_{12}, is marked by a dashed circle. X: upper ligand.

novo (e.g. *Desulfitobacterium hafniense* strain Y51, Firmicutes) (Nonaka *et al.*, 2006; Reinhold *et al.*, 2012) and others depending strictly on corrinoid salvaging from the environment (e.g. *Dehalococcoides mccartyi*, Chloroflexi) (Löffler *et al.*, 2013). A recent study from Taga and colleagues (Yi *et al.*, 2012) showed the reductive dehalogenation of trichloroethene (TCE) by *D. mccartyi* strain 195 to be dependent on the availability of DMB, 5-methylbenzimidazole or 5-methoxybenzimidazole as lower ligand bases, while e.g. adenine could not replace these benzimidazole analogues in this respect. Such benzimidazolyl cobamides might be provided by other bacterial species in the natural environment (Yan *et al.*, 2012; 2013).

The Gram-negative Epsilonproteobacterium *Sulfurospirillum multivorans* utilizes hydrogen, formate or pyruvate as electron donors for the reductive dehalogenation of tetrachloroethene (PCE) via TCE to *cis*-1,2-dichloroethene (*c*DCE) in its energy metabolism (Scholz-Muramatsu *et al.*, 1995). The key enzyme PCE reductive dehalogenase (PceA), which contains two tetranuclear iron–sulfur clusters and a cobamide as cofactors, is located at the periplasmic face of the cytoplasmic membrane (for a tentative scheme of the PCE respiratory chain, see John *et al.*, 2006). The cytoplasmic precursor of the enzyme (prePceA) bears an N-terminal twin-arginine translocation (Tat; Palmer and Berks, 2012) signal peptide for export across the cytoplasmic membrane. The unusual norpseudo-B_{12} (Co_{α}-adeninyl-176-norcobamide) (Fig. 1A) of the PceA in *S. multivorans* is the only cobamide cofactor of a reductive dehalogenase characterized so far (Kräutler *et al.*, 2003). Like the more common cobamide cofactor pseudo-B_{12} (Dion *et al.*, 1952), the norpseudo-B_{12} of PceA harbours an adenine moiety as lower ligand base. However, the latter differs from pseudo-B_{12} by the absence of a methyl group bound to carbon 176 in the aminopropanol phosphate linker of the nucleotide loop (Fig. 1A). It has been suggested that the methyl group at position 176 favours the '*base-on*' conformation state, in which the lower ligand coordinatively binds to the cobalt (Butler *et al.*, 2006). In norcobamides, lacking the methyl group, the formation of the '*base-off* non-coordinated state of the ligand is facilitated.

In this study, the specificity of the *S. multivorans* PceA enzyme for the norpseudo-B_{12} cofactor was tested by re-routing the *de novo* corrinoid biosynthesis. The presence of exogenous DMB (Fig. 1B) resulted in the formation of nor-B_{12} (Co_{α}-5,6-dimethylbenzimidazolyl-176-norcobamide) and affected PCE-dependent growth of the organism. The role of the cobamide cofactor's lower ligand base in the biosynthesis and reactivity of the PceA in *S. multivorans* was investigated.

Results

PCE-dependent growth in the presence of exogenous DMB

PCE-dependent growth of *S. multivorans* was shown to be independent on supplementation of the growth medium with vitamin B_{12} (Siebert, 2002) indicating a functional *de novo* corrinoid biosynthesis in the organism. Cultivation of *S. multivorans* in the presence of vitamin B_{12} has been tested earlier, and neither a promoting nor an inhibiting effect on the growth with PCE as electron acceptor was observed (data not shown). Total sequencing of the *S. multivorans* genome revealed a gene cluster encoding proteins for *de novo* corrinoid biosynthesis (T. Goris, T. Schubert, T. Wubet, M. Tarkka, L. Adrian and G. Diekert, unpubl. data). To investigate the effect of DMB as alternative lower ligand base on growth and PCE dechlorination, all cultivations described in this study were conducted without the addition of exogenous vitamin B_{12}. The cells were grown with pyruvate as electron donor and either PCE or fumarate as electron acceptor. With fumarate as electron acceptor, PceA is still formed for several subcultivations; however, the enzyme is not required for growth (John *et al.*, 2009).

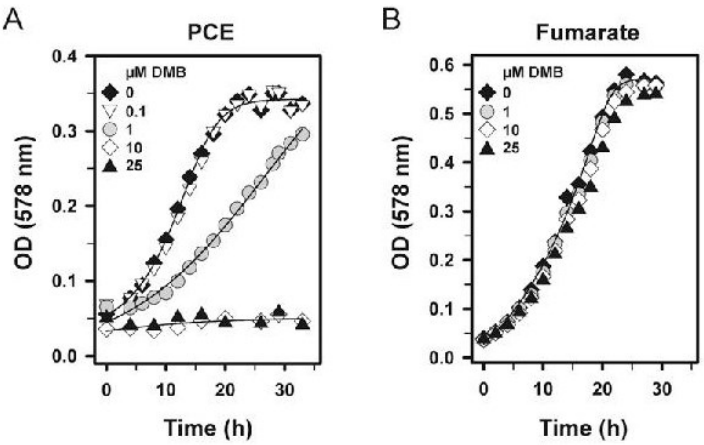

Fig. 2. Growth of *S. multivorans* in the absence and presence of DMB.
A. Cultivation with tetrachloroethene (PCE) as terminal electron acceptor.
B. Growth with fumarate instead of PCE.
The DMB concentrations (μM) are given in the figure.

DMB concentrations up to 0.1 μM had no effect on growth rate and yield with PCE as electron acceptor and on PCE conversion to TCE and *c*DCE. However, higher DMB concentrations affected growth with PCE significantly (Fig. 2A). To exclude a general effect of DMB on the viability of the organism, *S. multivorans* was also cultivated in pyruvate/fumarate medium in the presence of DMB (Fig. 2B). No effect of DMB on growth with fumarate was observed. The results pointed to an effect of DMB on the reductive dechlorination of PCE, possibly caused by biosynthesis of an alternative cobamide cofactor in *S. multivorans*.

Activity and processing of the PceA enzyme

The PceA enzyme activity was determined in cells grown with PCE in the absence or presence of DMB. The addition of up to 0.1 μM DMB to the growth medium had no drastic effect on the PceA enzyme activity (activity decrease < 10%); in the presence of 1 μM DMB, the activity was significantly reduced (Fig. 3). In PCE-grown cells, the activity could not be tested with higher DMB concentrations because of the low cell yields. The presence of 10 or 25 μM DMB during growth with fumarate did not result in a complete loss of the PceA activity (decrease > 80%), although growth with PCE was severely inhibited at these DMB concentrations. DMB (25 μM) did not have an inhibitory effect on the PceA activity in the enzyme assay, which excludes a direct effect of DMB on PceA (data not shown).

An explanation for the results may be an effect of DMB either on the synthesis of PceA or on the localization of the enzyme or both. In cells grown with PCE, a largely periplasmic localization of PceA has been reported (John *et al.*, 2006), which is supposed to be essential for the function of the enzyme in organohalide respiration. Hence, it is feasible that DMB has an impact on the maturation and/or export of the enzyme, thus affecting functional PCE respiration. This was tested by immunoblot analysis with antibodies directed against PceA in pyruvate/fumarate-grown cells (Fig. 4). Using this method, it is possible to discriminate between the mature PceA and the unprocessed precursor (prePceA) (John *et al.*, 2006). The band with the higher molecular mass represents the prePceA (57.1 kDa) bearing the N-terminal

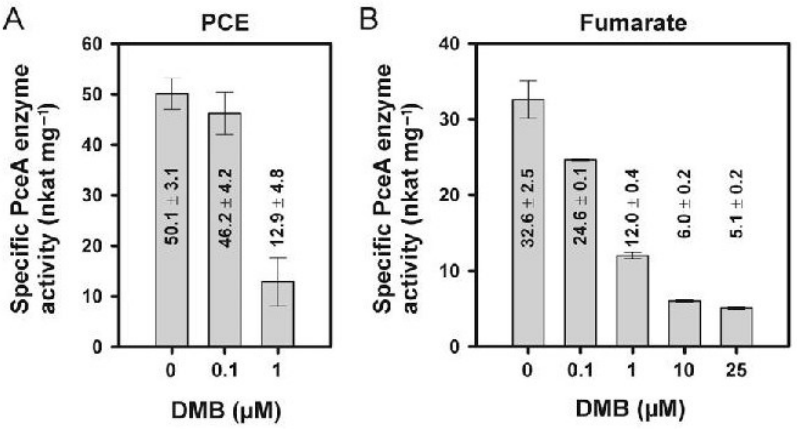

Fig. 3. Specific PceA enzyme activity (nkat mg^{-1}) in crude extracts from cells grown in the absence and presence of DMB.
A. Cultivation with tetrachloroethene (PCE) as terminal electron acceptor.
B. Growth with fumarate instead of PCE.
The average of at least two cultures with the maximum deviation is displayed.

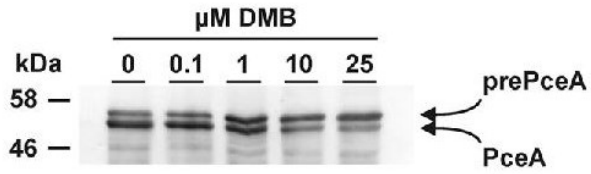

Fig. 4. Immunological analysis of the PceA protein in crude extracts from cells grown on pyruvate and fumarate with different concentrations of DMB. Five micrograms of protein were applied to each lane. The DMB concentrations are given above each lane. prePceA, precursor of the PCE reductive dehalogenase bearing the Tat signal peptide; PceA, processed form of the enzyme without the Tat signal peptide.

Tat signal peptide essential for membrane export, the band with the lower molecular mass represents the mature form of the enzyme (PceA, 53.3 kDa) without the signal peptide. The latter is cleaved off during membrane translocation.

Cells grown with increasing concentrations of DMB showed essentially an increasing ratio of unprocessed versus processed protein (Fig. 4); the total amount of PceA seemed to be unaffected. Apparently, DMB had an inhibitory effect on the PceA export.

Corrinoid biosynthesis in DMB-treated cells

The negative effect of DMB on the PceA activity (Fig. 3) pointed to an uptake of DMB and use as lower ligand base precursor in the corrinoid cofactor biosynthesis. In order to identify the cobamides produced in the absence and presence of DMB, they were extracted from the cells, purified and analyzed by high-performance liquid chromatography (HPLC) (Fig. 5). The cobamide extraction was conducted in the presence of KCN, resulting in the binding of a cyano group as upper ligand to the cobalt of the purified cobamides. The cyano-cobamides are designated by the term 'vitamin' included in the trivial name. When no DMB was present during growth of *S. multivorans*, a single peak was detected in the HPLC elution profile that represented the norpseudovitamin B_{12} (Co_{β}-cyano-form of norpseudo-B_{12}) previously described (Kr utler *et al*., 2003). With increasing DMB concentrations, a second cobamide peak was visible that became predominant at ≥ 1 µM DMB in the medium. From the characteristic absorbance spectrum of this compound, monitored using a diode array detector, it could be identified as a cobamide. The total amount of cobamides produced by the cells (~ 1.4 µmol per g protein) was not influenced by DMB.

Using electrospray ionization mass spectrometry, the unknown cobamide was further analyzed and displayed a pseudomolecular ion $[M + H]^+$ at *m/z* 1341.3 (see Supporting Information Fig. S1) that is comparable with the mass-to-charge ratio reported by Butler and colleagues (2006) for synthetic norvitamin B_{12} (Co_{α}-5,6-dimethylbenzimidazolyl-Co_{β}-cyano-176-norcobamide; *m/z* 1341.5). The ^{1}H-NMR spectrum of the isolated cobamide was identical to that of a synthetic sample of norvitamin B_{12} showing the characteristic signals of the methylene protons of the ethanolamine linker at 3.26/3.64 and 3.94/4.04 p.p.m. (see Supporting Information Fig. S2). Furthermore, the doublet signal in the ^{1}H-NMR spectrum of vitamin B_{12} at 1.23 p.p.m. corresponding to the C-177 methyl group was missing. These results supported the assumption that in the presence of exogenous DMB in concentrations > 0.1 µM, this base is incorporated during cobamide biosynthesis to form nor-B_{12} as a product that might lead to the production of a functionally affected enzyme.

Enrichment of PceA from DMB-treated S. multivorans cells

Two explanations for the reduced dechlorinating activity in DMB-treated cells were feasible: (i) a low affinity of the precursor of the PCE reductive dehalogenase (prePceA) for nor-B_{12} which inhibits the incorporation of the cofactor into the apoprotein and (ii) a low reactivity of nor-B_{12} against organohalides. To test for the first possibility, the PceA was purified from cells grown in the absence or presence of 25 µM DMB and analyzed with respect to the cobamide cofactor incorporated. PrePceA and the mature

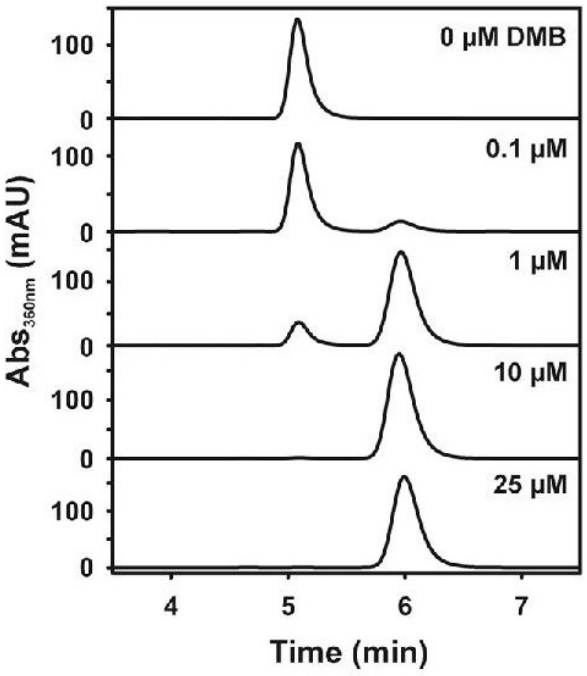

Fig. 5. HPLC analysis of cobamide extracts from cells cultivated with different DMB concentrations. For experimental details, see the *Experimental procedures* section.

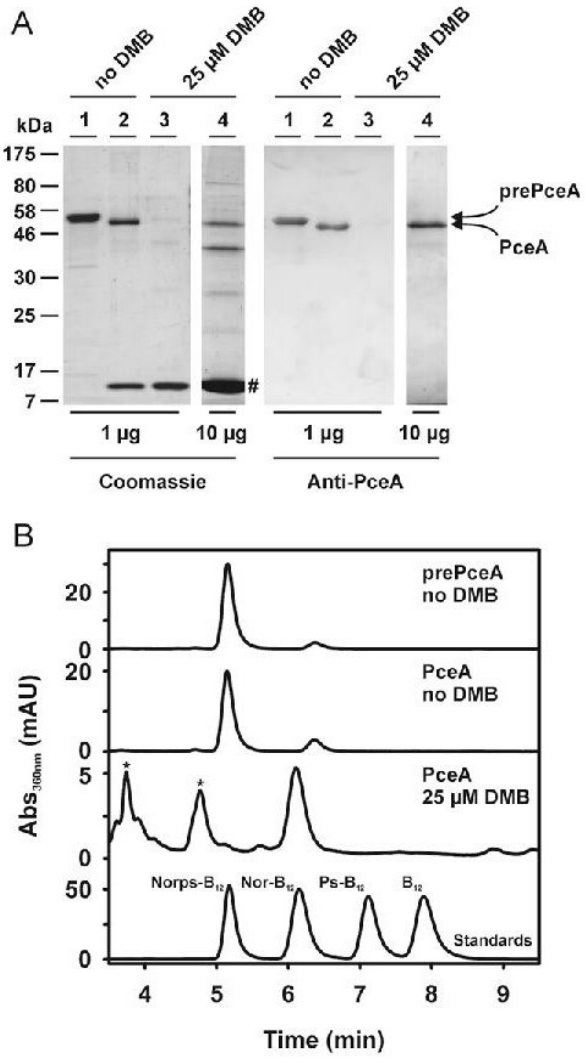

Fig. 6. Extraction of the cobamide cofactor out of the PCE reductive dehalogenase enriched from cells grown in the absence or presence of 25 µM DMB.
A. Analysis of the enzyme containing fractions via Coomassie-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot developed with an antibody against PceA. The amount of protein applied is given below the lanes. Lane 1: purified prePceA, lane 2: enriched PceA, lanes 3 and 4: PceA containing fraction enriched from DMB-treated cells, # marks a protein related to pyridoxamine 5'-phosphate oxidase.
B. HPLC analysis of the extracted cobamide cofactor from the PceA containing fractions. The standard mix contained Norps-B_{12}: norpseudovitamin B_{12}, Nor-B_{12}: norvitamin B_{12}, Ps-B_{12}: pseudovitamin B_{12}, and B_{12}: vitamin B_{12}. The asterisks mark impurities in the cobamide containing fraction that did not display the characteristic cobamide absorbance spectrum (data not shown).

form of the enzyme (PceA) were enriched from cells cultivated in the absence of DMB. The cobamides were extracted from prePceA (Fig. 6A, lane 1; specific enzyme activity 1226 nkat mg^{-1}) and PceA (Fig. 6A, lane 2; specific activity of PceA in this fraction 2386 nkat mg^{-1}) containing fractions. The cobamide analysis is shown in Fig. 6B. The extracted cobamide cofactor of prePceA and of PceA enriched from cells grown without DMB was identified as norpseudovitamin B_{12} (Co_{β}-cyano-form of norpseudo-B_{12}). From cells grown in the presence of 25 µM DMB, significantly less PceA was obtained (Fig. 6A, lane 3; specific activity of PceA in this fraction 64 nkat mg^{-1}). Using the purification procedure described, no prePceA could be enriched from such cells. The cobamide extracted from PceA was identified as norvitamin B_{12} (Co_{β}-cyano-form of nor-B_{12}) (Fig. 6B). The determination of the stoichiometry cobamide cofactor per PceA molecule was only possible for prePceA purified from cells grown in the absence of DMB. According to this quantification, about 100% of prePceA contained a cobamide cofactor. PceA was not purified to apparent homogeneity; more than 50% of the total protein in the fraction containing PceA (Fig. 6A, lane 2) was a second protein identified by peptide mass fingerprinting as a flavin mononucleotide-binding protein related to pyridoxamine 5'-phosphate oxidase (data not shown). Such proteins are not known to harbour a cobamide cofactor. Based on the results from cobamide extraction and quantification, a 1:1 stoichiometry was estimated for PceA (data not shown), taking into account that at most 50% of the protein in the fraction was the PceA. From the PceA-containing fraction obtained from DMB-treated cells, only traces of nor-B_{12} cofactor were extracted. Because the impurity in these fractions was considerably higher than 50% (Fig. 6A, lanes 3 and 4), even a rough estimation of the stoichiometry cobamide cofactor per PceA was not possible. The fact that only very low amounts of PceA can be enriched from DMB-treated cells points to an impeded maturation of the enzyme, i.e. a retarded incorporation of the nor-B_{12} cofactor into the apoprotein.

The low dechlorination activity of DMB-treated cells may also be due to a significantly lower activity of PceA containing nor-B_{12} than PceA containing norpseudo-B_{12}. Because PceA harbouring nor-B_{12} cannot be obtained in sufficient amount and purity to determine a turnover number (mol Cl^- released per mol of PceA per second), we tested and compared the abiotic dechlorination activity of the purified cofactors with trichloroacetate (TCA) as substrate using the same assay as that used for PceA (Fig. 7) (Neumann *et al.*, 2002). In this test, PCE was not applied because of the low abiotic activity of cobamides with this organohalide. Norpseudovitamin B_{12} showed the highest turnover rate of TCA as reported before by Neumann and colleagues (2002). Norvitamin B_{12} exhibited only about 5% of the dechlorination activity measured with norpseudovitamin B_{12}. Pseudovitamin B_{12} (Co_{β}-cyano-form of pseudo-B_{12}) and vitamin B_{12} (Co_{β}-cyano-form of coenzyme B_{12}) with the methyl group at position

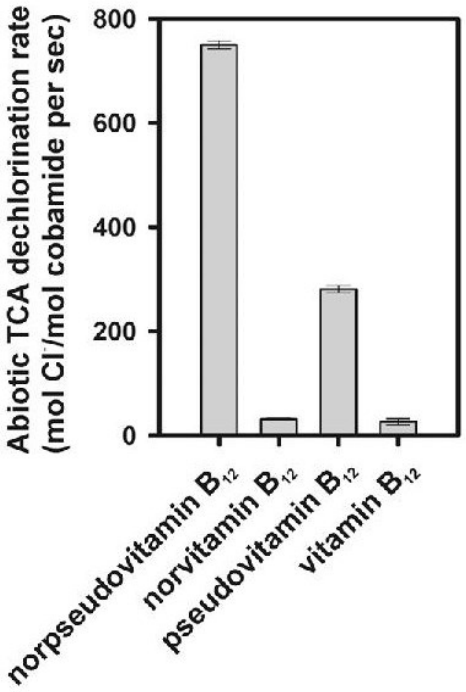

Fig. 7. Abiotic dechlorination of trichloroacetate (TCA) by different cobamides. The average of at least two cobamide extracts measured in duplicate with the maximum deviation is displayed.

176 in the linker moiety displayed a significantly reduced rate, a result pointing to the impact of the methyl group at position 176 for the reactivity.

Discussion

Several different cobamide-producing prokaryotes synthesize and use pseudo-B_{12} (Co_{α}-adeninyl-cobamide) (summarized in Taga and Walker, 2008), which harbours an adenine moiety as lower ligand base. In *Salmonella enterica* serovar typhimurium, the adeninylcobamide could be replaced by 5,6-dimethylbenzimidazolyl-cobamide by addition of DMB to the growth medium without affecting corrinoid-dependent enzymatic reactions (Anderson *et al.*, 2008). This observation pointed to flexibility of the use of cobamides in *S. enterica*. In contrast, for the acetogen *Sporomusa ovata*, a strict dependency of the cobamide-dependent methylotrophic metabolism on the *p*-cresolyl or phenolyl cobamides synthesized by this organism was reported (Mok and Taga, 2013). In this report, the incorporation of DMB as lower ligand base into the cobamide produced by the reductively dehalogenating *S. multivorans* was shown to result in a reduced growth of the organism with PCE as terminal electron acceptor and a significant decrease of the cobamide-dependent PceA enzyme activity in crude extracts. This result implied a limited flexibility of this reductive dehalogenase with respect to its cofactor and might even indicate a preference for an adeninylcobamide that has not been reported before for other cobamide-dependent enzymes.

In the cobamide biosynthesis gene cluster of the *S. multivorans* genome, all genes have been identified which encode the proteins supposed to mediate the activation and incorporation of the lower ligand base (T. Goris, T. Schubert, T. Wubet, M. Tarkka, L. Adrian, G. Diekert, unpubl. data). For the cobamide biosynthesis in other bacteria (e.g. *S. enterica* serovar typhimurium), it was shown that different bases can be incorporated by the corresponding enzymes (Anderson *et al.*, 2008). Hence, it is feasible that these corrinoid biosynthesis enzymes in *S. multivorans* also display a broad spectrum for the base that is incorporated. Amendment of the growth medium with up to 10 μM *p*-cresol, however, had no effect on the growth and the synthesis of norpseudo-B_{12} (data not shown). Either this lower ligand is not taken up or it is not incorporated into the cobamides by *S. multivorans*.

Apparently, *S. multivorans* is not able to synthesize DMB *de novo* so that adenine is used as a base in corrinoid biosynthesis when no DMB is present in the medium. The addition of DMB leads to an uptake and incorporation of this base, dependent on its concentration, an effect reported earlier for other cobamide-producing bacteria and designated as corrinoid 'guided biosynthesis' (Renz, 1999). At high DMB concentrations, the intracellular DMB might outcompete the adenine in cobamide biosynthesis, which results in the formation of an increasing portion of significantly less active cobamide cofactor for PceA. In DMB-treated cells, the total amount of cobamides and of PceA appears to be unaffected, whereas the PceA activity is significantly reduced. Either nor-B_{12} formed in the presence of DMB is incorporated into the PceA apoprotein leading to a malfunction of the enzyme or nor-B_{12} is not incorporated at all. To discriminate between these two possibilities is difficult because a non-functional, possibly malfolded enzyme cannot be easily purified.

DMB-treated cells produced by far less functional PceA than cells grown in the absence of DMB and the growth rates were lower. The total amount of PceA as shown by immunoblot in Fig. 4 as well as that of cobamides was not reduced in DMB-amended cells. This points to a severe effect of DMB on PceA maturation. For cobamide analysis, the PceA enzyme produced in cells of *S. multivorans* grown in the presence of 25 μM DMB was enriched and shown to harbour nor-B_{12}. The low concentration of functional PceA in such cells obviously limited growth with PCE as well as the enzyme activity in crude extracts. In addition, it is feasible that the enzyme harbouring nor-B_{12} rather than norpseudo-B_{12} has no or has a reduced dechlorinating activity. The specific activity of the nor-B_{12}-

containing enzyme could not be determined in this study. However, the observation of a significantly lower abiotic TCA dechlorination activity of norvitamin B_{12} in comparison with norpseudovitamin B_{12} supports this assumption.

In the reductively dehalogenating *Geobacter lovleyi* and *D. mccartyi* strain 195, no inhibition of the organohalide-dependent growth by DMB was observed (Yan *et al.*, 2012; Yi *et al.*, 2012). Whereas *G. lovleyi* is able to synthesize corrinoids *de novo*, *D. mccartyi* strain 195 is strictly dependent on the presence of DMB plus cobinamide or vitamin B_{12} in the culture medium because this organism is lacking the capability of *de novo* corrinoid biosynthesis (L ffler *et al.*, 2013). This shows that in principle reductive dechlorination is also possible with cobamide cofactors other than norpseudo-B_{12} in other organisms.

Experimental procedures

Cultivation of bacterial cells

Sulfurospirillum multivorans (DSMZ 12446) was grown anoxically at 28°C in a defined mineral medium (Scholz-Muramatsu *et al.*, 1995) without vitamin B_{12} (cyanocobalamin) and yeast extract. Where indicated, a sterile 1 mM DMB stock solution in ultrapure water (UPW) was added prior to inoculation of the cultures. Pyruvate (40 mM) was used as electron donor and either fumarate (40 mM) or PCE (10 mM nominal concentration; stock solution 0.5 M PCE dissolved in hexadecane) as electron acceptor. The serum glass bottles used for cultivation were capped with Teflon-coated butyl rubber septa. Growth was monitored photometrically by measuring the optical density at 578 nm. For each growth experiment depicted in Fig. 2, two subsequent pre-cultures were grown under conditions identical to those of the growth experiment. The second pre-culture served as inoculum (10%) for two parallel cultures in the growth experiment; inoculation was performed when the culture without DMB addition was in the late exponential growth phase. The experiment was repeated to ensure reproducibility. Growth curves were plotted using average values of the optical densities of the different cultures. For strain maintenance, *S. multivorans* was cultivated on pyruvate/PCE-containing medium.

PceA activity measurements

Cells from a 100 ml anoxic culture were harvested aerobically (10 min, 12 000 × g, 4°C) at an OD (578 nm) of approximately 0.3 when grown on pyruvate/PCE or at an OD (578 nm) between 0.5 and 0.6 when grown on pyruvate/fumarate. The cell pellet was immediately transferred into an anaerobic chamber and resuspended in anoxic buffer (50 mM Tris-HCl, pH 7.5; per gram wet cells 2 ml buffer). The cell suspension was mixed with an equal volume of glass beads (0.25–0.5 mm diameter, Carl Roth GmbH, Karlsruhe, Germany). The cells were disrupted using a beadmill (5 min at 25 Hz; Mixer Mill MM400, Retsch GmbH, Haan, Germany). Crude extract and glass beads were separated by centrifugation (2 min, 2000 × g) under anoxic conditions. The PceA was assayed according to the procedure described elsewhere (Neumann *et al.*, 1996). For determination of the protein concentration, the method of Bradford (1976) was applied using the Roti-Nanoquant reagent (Carl Roth GmbH).

Purification of the PceA

The PceA enzyme was purified by a procedure described by Neumann and colleagues (1996). This procedure comprised a Q-Sepharose, Phenyl Superose and Superdex 75 pg chromatography step. The final step of the protocol was modified. The previously described size exclusion chromatography was replaced by an anion exchange chromatography step. The PceA activity containing fractions from the Phenyl Superose chromatography were pooled and diluted in basal buffer (50 mM HEPES, pH 7.5, 0.5 mM DTT) to a final concentration of 10 mM ammonium sulfate. The solution was applied to a Mono Q column (1 by 10 cm) pre-equilibrated with basal buffer. The enzyme was eluted in a linear gradient from 0 to 0.5 M NaCl. The prePceA eluted at 0.15 M NaCl and the mature form (PceA) at 0.25 M NaCl.

Immunoblot analysis

Crude extracts of *S. multivorans* (5 μg protein/lane) or purified PceA enzyme (1 or 10 μg protein/lane) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (12.5%). The immunological analysis was conducted as described earlier (John *et al.*, 2009). The PceA antiserum was diluted 50 000-fold, and the antibodies were detected via a secondary antibody coupled to alkaline phosphatase (Sigma-Aldrich, Munich, Germany).

Cobamide purification

After three subsequent cultivations of *S. multivorans* on pyruvate/fumarate medium containing the DMB concentrations as indicated, cells were harvested by centrifugation (10 min, 6700 × g, 10°C); ~ 8 g wet cells were resuspended in 24 ml 50 mM Tris-HCl (pH 7.5). The cells were disrupted in a French pressure cell at 2000 psi (French Pressure Cell Press, Sim-Aminco, Spectronic Instruments, Rochester, NY, USA). Cobamide extraction and purification was performed according to the protocol described by Stupperich and colleagues (1986). The pH of the crude extract was adjusted to ≤ 5 with acetic acid. Subsequently, 0.1 M potassium cyanide was added. The sample was boiled for 15 min. After centrifugation of the sample, the supernatant containing the cobamides was kept on ice, and the pellet was resuspended in 10 ml UPW. The resuspended pellet was subjected to the same extraction procedure twice. The combined supernatants from all three extractions were mixed with Amberlite XAD4 (0.25 g XAD4 per ml extract; Sigma-Aldrich) and incubated on a shaker overnight. After sedimentation of the cobamide-loaded XAD4 material, the supernatant was removed. The XAD4 material was washed with 10 volumes of UPW. Subsequently, the cobamides were eluted with one volume of methanol (1 h incubation on a shaker). The elution was repeated twice. The cobamide-containing eluates were

dried completely using a vacuum concentrator. The dry sample was resuspended in 2 ml UPW and transferred to a column containing 3 g aluminium oxide. The cobamides were recovered from the column by washing with at least 40 ml UPW. The eluate was dried as described above, and the pellet was resuspended in 100 µl UPW. When the cobamide cofactor was extracted directly from the purified or enriched PceA enzyme, the same procedure was applied. The cobamide-dependent abiotic dechlorination of TCA was assayed as previously described by Neumann and colleagues (2002). Methyl viologen (0.5 mM) reduced with Ti(III) citrate was used as electron donor and TCA (1 mM) served as electron acceptor in the assay. The amount of each cobamide applied to a measurement was between 1 and 10 pmol.

Analysis of the cobamides

The purified cobamides were analyzed using HPLC (Smartline System, Knauer GmbH, Berlin, Germany) combined with a diode array detector. A reverse phase column was used (Chromolith Performance, RP-18e, 100-4.6 mm, Merck, Darmstadt, Germany) with a flow rate of 1 ml min^{-1} at 30°C. Mobile phases used were 18% methanol/0.2% acetic acid (solvent A) and 99.8% methanol/0.2% acetic acid (solvent B). The cobamides were separated by 0–10 min solvent A, followed by a gradient to 100% solvent B within 4 min, and finally 100% solvent B for 3 min. To ensure reproducibility every HPLC analysis included the application of a mix of cobamide standards (10 µM each) containing norpseudovitamin B_{12}, norvitamin B_{12} (both purified from *S. multivorans*), pseudovitamin B_{12} (purified from *Propionibacterium acidipropionici* according to Hoffmann *et al.*, 2000) and vitamin B_{12} (Sigma-Aldrich).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Base peak region of the ESI mass spectrum of the isolated cobamide (spectrum A) and calculated mass spectra of norvitamin B_{12} (spectrum B), vitamin B_{12} (spectrum C) and norpseudovitamin B_{12} (spectrum D). The spectra were simulated for the protonated molecular ion $[M + H]^+$ and the sodium adduct $[M + Na]^+$ respectively.

Fig. S2. Section of the 500 MHz ^{1}H-NMR spectra (D_2O, 25°C) of synthetic norvitamin B_{12} (spectrum A, blue) and of the isolated cobamide (spectrum B, red). The signals of the four H-atoms attached at the ethanolamine linker are highlighted with arrows. (X indicates residual solvent signals)

Exogenous 5,6-dimethylbenzimidazole caused production of a non-functional tetrachloroethene reductive dehalogenase in *Sulfurospirillum multivorans*

Sebastian Keller[1], Markus Ruetz[2], Cindy Kunze[1], Bernhard Kräutler[2], Gabriele Diekert[1], and Torsten Schubert[1#]

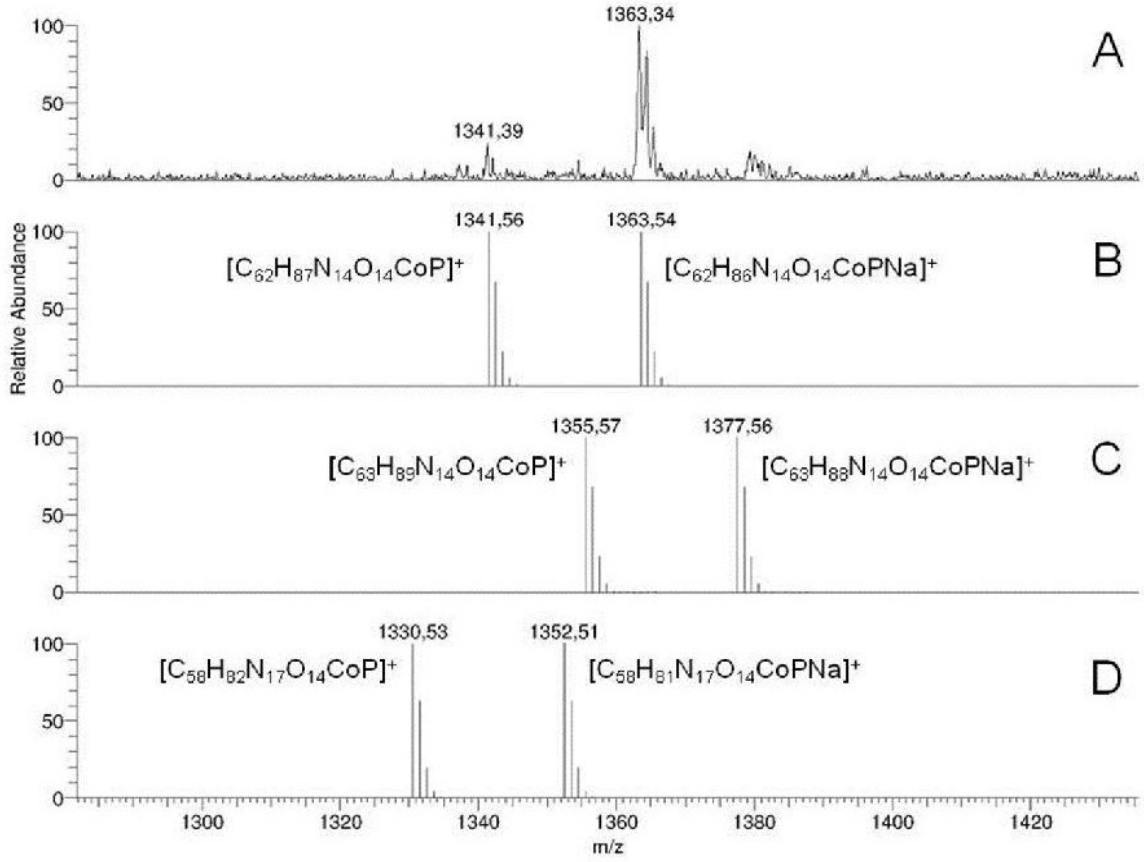
Supporting Information

General.

Materials. Synthetic norvitamin B_{12} was prepared as described in ref [S1], D_2O (99.96% D) was from Eurisotop.

Spectroscopy. *^{1}H-NMR spectra:* 500 MHz Varian Unity Inova equipped with 5 mm triple-resonance probe with z-gradients; δ(HDO) = 4.77 ppm; *ESI-MS*: Finnigan LCQ classic, positive-ion mode, spray voltage 4.3 kV, solvent MeOH.

Sample preparation for NMR experiment. A sample of about 70 µg of the isolated cobamide was dissolved in 100 µl D_2O and was dried under high vacuum overnight. The residue was dissolved in 300 µl D_2O and transferred into a Shigemi NMR tube (Shigemi Inc.). The ^{1}H-NMR experiment was carried out at 25°C using water suppression techniques.



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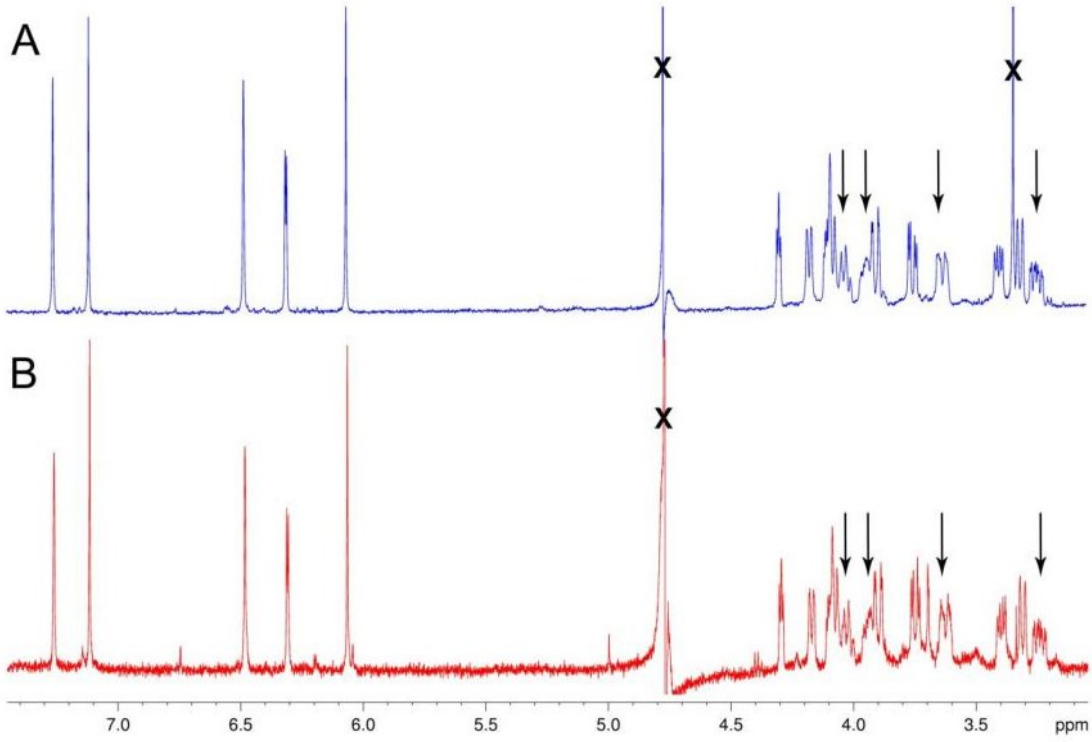

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[S1] Butler, P., Ebert, M.O., Lyskowski, A., Gruber, K., Kratky, C., and Kräutler, B. (2006) Vitamin B_{12}: A Methyl Group without a Job? Angew. Chem. Int. Ed. 45:989-93.

2.2 Molecular inside view of how benzimidazoles steer cobamide cofactor biosynthesis and utilization in the organohalide-respiring bacterium *Sulfurospirillum multivorans*

Sebastian Keller, Cindy Kunze, Martin Bommer, Christian Paetz, Riya C. Menezes, Aleš Svatoš, Holger Dobbek, Torsten Schubert

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The exogenous benzimidazoles Bza, 5-MeBza, 5-OMeBza, or 5-OHBza at 25 µM efficiently replaced the adenine of norpseudo-B_{12} in *S. multivorans*, which had no significant effect on the PCE-metabolism in the cases of Bza, OMeBza, and OHBza due to an effective incorporation of the respective norcobamides into PceA at the same position like norpseudo-B_{12}. The regioselective enzyme *Sm*CobT synthesized a mixture of 5- and 6-(Bza) α-ribotides (α-RP), mainly 5-OMeBza-, 5-MeBza-, and 6-OHBza α-RP from the singly substituted benzimidazoles *in vitro*. However, only 5-OMeBza-Nba, 6-OHBza-NCba, and 5- and 6-MeBza-NCba were formed in the cell revealing a selectivity for certain CobT products in *S. multivorans*. Both, 5- and 6-MeBza-NCba were incorporated into PceA, which did not affect the PCE-dependent growth, but resulted in a reduced activity and processing of the enzyme pointing towards a low compatibility of PceA and cobamides harboring methylated benzimidazoles.

Contribution of Sebastian Keller to this study: 50 %

Sebastian Keller performed the cobamide extractions and HPLC analysis of *S. multivorans* amended with the benzimidazoles (Fig. 1 B,C) and prepared the MS analysis. He performed the heterologous expression and CobT purification plus HPLC analysis and prepared the MS analysis of the CobT products (Fig. 3; 4 A-C). He performed the growth studies and determination of PceA activity and maturation in crude extracts of *S. multivorans* amended with the benzimidazoles (Fig. 5) and contributed to the writing of the manuscript.

Molecular inside view of how benzimidazoles steer cobamide cofactor biosynthesis and utilization in the organohalide-respiring bacterium *Sulfurospirillum multivorans*

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2.2 Biosynthesis and utilization of benzimidazolyl norcobamides in *S. multivorans*

SUMMARY

Structural variability in natural cobamides is limited, but expected to be crucial for the functionality of selected cobamide-containing enzymes. In this study, the susceptibility of the organohalide-respiring bacterium *Sulfurospirillum multivorans* for guided biosynthesis of various benzimidazolyl-norcobamides was investigated and their use as a cofactor of the tetrachloroethene reductive dehalogenase (PceA) was analyzed. Exogenously applied benzimidazole (Bza), 5-methylbenzimidazole (5-MeBza), 5-hydroxybenzimidazole (5-OHBza), and 5-methoxybenzimidazole (5-OMeBza) besides the previously investigated 5,6-dimethylbenzimidazole (DMB) were found to be efficiently incorporated as lower bases into norcobamides. The *in vitro* enzymatic activation of the singly substituted 5-benzimidazoles by the organism's benzimidazole phosphoribosyltransferase (CobT) lead to the formation of both, the 5- and the 6-substituted α-ribotide isomer in all cases. However, mass analysis and nuclear magnetic resonance spectroscopy revealed an almost exclusive incorporation of 5-OMeBza or 6-OHBza α-ribotide, but a non-selective one of 5- and 6-MeBza α-ribotide into the final norcobamide cofactors. In crystal structures of PceA, the cofactors were bound in 'base-off' mode with 6-OHBza and 5-OMeBza lower bases accommodated by a solvent-exposed hydrophilic pocket. Thus, PceA of *S. multivorans* is the first cobamide-dependent enzyme for which structures containing adenine- and benzimidazole-harboring cobamide cofactors are available. Exclusively when 5-MeBza was added to cultures the PceA enzyme activity was affected, which resembled the previously reported incompatibility of PceA with (DMB), the lower base of cobalamin (vitamin B_{12}).

INTRODUCTION

The type of axial ligands bound to the central cobalt ion in cobamide (Cba) cofactors differs among the various B_{12}-containing enzymes and reflects their functional diversity (Banerjee and Ragsdale 2003, Gruber *et al.* 2011, Bridwell-Rabb and Drennan 2017). Cba cofactors vary in their structure by harboring different lower bases, either purines or benzimidazoles (Renz 1999). Two different pathways have been described for the biosynthesis of benzimidazoles: the direct formation of DMB from flavin in an oxygen-dependent reaction (Taga *et al.* 2007) and the anoxic formation of DMB via 5-OHBza, 5-OMeBza, and 5-methoxy-6-

methylbenzimidazole from 5-aminoimidazole ribotide (Hazra *et al.* 2015), an intermediate of the purine biosynthesis. Prior to their incorporation into Cbas, the various benzimidazoles are activated by the CobT enzyme (nicotinate mononucleotide:5,6-dimethylbenzimidazole phosphoribosyltransferase, EC 2.4.2.21), which adds a phosphoribosyl moiety and forms α-ribotides (Trzebiatowski and Escalante-Semerena 1997, Claas *et al.* 2010, Hazra *et al.* 2013, Crofts *et al.* 2013, Chan *et al.* 2014). Subsequently, the α-ribotides are added to cobinamide guanosine diphosphate (Cbi-GDP), a late intermediate of the Cba biosynthetic pathway, by CobS (adenosylcobamide synthase, EC 2.7.8.26) and finally dephosphorylated by CobC (adenosylcobalamin/alpha-ribazole phosphatase, EC 3.1.3.73) (summarized in Escalante-Semerena 2007). The relaxed specificity of some CobTs allowed for the formation of uncommon 6-substituted benzimidazolyl-ribotides from singly substituted benzimidazoles such as 5-OHBza or 5-OMeBza, which can lead to the formation of unusual Cba isomers (Crofts *et al.* 2014). Friedrich and Bernhauer (1958) reported the extraction and identification of 5-MeBza-Cba and 6-MeBza-Cba produced by *Propionibacterium shermanii* in the presence of 5-MeBza, albeit in a ratio of 96:4, which showed a clear preference for the formation of 5-MeBza-Cba in this organism. In *Methanobacterium thermoautotrophicum*, which has been shown to produce OHBza-Cba, approximately 10 % of 6-OHBza-Cba was detected (Kräutler *et al.* 1986). Recently, Taga and coworkers found a mixture of Cba isomers in *Sinorhizobium meliloti* and *Veillonella parvula*, both cultivated in the presence of 5-OMeBza or 5-OHBza (Crofts *et al.* 2013, Crofts *et al.* 2014). However, the exclusive biosynthesis of benzimidazolyl-Cbas with a substituent in position 6 of the benzimidazolyl-moiety has not been described so far. The lower ligand base, which is part of the Cba's nucleotide loop and thereby covalently bound to the cofactor, can be displaced from the cobalt ion ('base-off') in the protein-bound state. An alternative lower ligand such as a histidine residue of the protein ('base-off/His-on') can fulfill this function (Ludwig and Matthews 1997, Buckel and Golding 2008). For Cba cofactors bound to reductive dehalogenases (RDase) a lower ligand of the cobalt was not detected by structural analysis (Bommer *et al.* 2014, Payne *et al.* 2015). These RDases harbor the Cba cofactor deeply buried inside the protein bound by a network of hydrogen bonds. In the RDase-bound state the lower base of the Cba is not placed in proximity to the cobalt, it is completely moved out of the position it obtains in the protein-free state of the cofactor. In case of the two known RDase structures, PceA of *S. multivorans* and *Np*RdhA of *Nitratireductor pacificus* pht-3B, binding of an alternative ligand is prevented by the protein structure. These

observations were confirmed by spectroscopic analyses of different RDases using electron paramagnetic resonance spectroscopy (EPR), which also detected the Cba cofactor in the 'base-off' state (Schumacher *et al.* 1997, van de Pas *et al.* 1999, Kräutler *et al.* 2003, Payne *et al.* 2015).

The unique norpseudo-B_{12} of the organohalide-respiring bacterium *Sulfurospirillum multivorans* (Kräutler *et al.* 2003, Schubert 2017) contains an adenine moiety as lower base. When DMB was added to cultures of *S. multivorans*, the DMB efficiently replaced the adenine in the norpseudo-B_{12}, which led to the formation of nor-B_{12} (Keller *et al.* 2014) in a process named guided biosynthesis (Renz 1999). The tetrachloroethene (PCE)-dependent growth, the PceA activity, and the PceA maturation was impaired in the presence of DMB. Negative effects on enzymatic reductive dehalogenation caused by the modification of the Cba structure have also been reported for *Dehalococcoides mccartyi* strains (Yi *et al.* 2012, Yan *et al.* 2012, Yan *et al.* 2013, Keller *et al.* 2014, Yan *et al.* 2015). The obligate organohalide-respiring *D. mccartyi* is a Cba auxotroph (Löffler *et al.* 2013). The dechlorination of trichloroethene (TCE) by *D. mccartyi* strain 195 was dependent on the availability of Cbas with either DMB, 5-MeBza, or 5-OMeBza as lower base. Cbas with alternative lower bases such as 5-OHBza or Bza did not sustain TCE-dependent growth of the organism. A plausible explanation for such inhibitory effects might be an incompatibility of a specific lower base in a Cba cofactor with the Cba binding site of a particular RDase. In order to shed light on the functionality of a single RDase with different Cba cofactors, we investigated the utilization of various benzimidazolyl-NCbas by PceA of *S. multivorans* and analyzed the similarities or variations in binding a Cba harboring either a purine or a benzimidazole as lower base. Thereby we identified PceA of *S. multivorans* as a norcobamide (NCba)-containing protein functioning with both, purinyl- and benzimidazolyl-norcoabamides, but limited in recruiting methylated benzimidazolyl-NCbas. Furthermore, we found evidence that the regioselectivity of the *S. multivorans* CobT is influenced by the type of substituent present in the singly substituted benzimidazole substrate.

RESULTS

Guided biosynthesis of benzimidazolyl-NCbas. In order to test the utilization of various benzimidazoles as lower bases for the production of NCbas (Fig. 1A), *S. multivorans* was

cultivated in the presence of DMB, 5-MeBza, Bza, 5-OMeBza, and 5-OHBza and the NCba fraction was extracted. The purified NCbas were applied to high performance liquid chromatography (HPLC) coupled to photometric detection (Fig. 1B-C). At increasing exogenous concentrations of the benzimidazoles the cobamide pool synthesized by *S. multivorans* contained decreasing portions of norpseudo-B_{12} besides novel cobamides (Fig. 1B-C) that shared characteristic Cba absorbance UV/Vis-spectra (data not shown). Ultra high performance liquid chromatography coupled with electrospray ionization high-resolution mass spectrometry (UHPLC-ESI-HRMS) was applied to these novel Cbas that were isolated from cells grown in the presence of 25 µM of the benzimidazoles (Fig. 1C, peak **1**-**5**). Due to an agreement with the predicted masses an incorporation of all tested benzimidazoles into norcobamides by *S. multivorans* was confirmed (Fig. 1C). The addition of DMB to the growth medium resulted in the formation of nor-B_{12} as it has been reported previously (Keller *et al.* 2014). After three subsequent cultivations in the presence of 25 µM exogenously applied benzimidazoles, the amount of norpseudo-B_{12} in the cells was reduced to a non-detectable level (Fig. 1B). Only in the case of 5-OMeBza, a residual amount (about 10 %) of norpseudo-B_{12} was observed. The NCba extract obtained from cells cultivated in the presence of 5-MeBza contained two NCbas (Fig. 1C, peak 1 and 2) with an identical mass as attributed to MeBza-NCba. The difference in the retention time of the two isomers might be caused by a difference in the position of the methyl group of the MeBza moiety in the NCba structure. In order to identify the compounds formed in the presence of singly substituted benzimidazoles NMR-analyses were conducted (see below). The total amount of Cba extracted from cells cultivated in the presence of benzimidazoles was not altered in comparison to untreated cells and was about 100 nmol NCba per gram wet cells.

The incorporation efficiency of all exogenous benzimidazoles was tested by extracting and analyzing the NCba fractions from cells cultivated in the presence of different concentrations of the lower base precursors (Fig. 1B). A strong divergence among the various benzimidazoles with respect to their appearance as lower bases in the NCba fraction was not observed, which indicates an efficient benzimidazole influx into the cells and enzymatic activation in all cases. The mechanism of benzimidazole uptake is not known so far. A curve for MeBza is not shown in Fig. 1B since NCba 1 (Fig. 1C) coeluted with norpseudovitamin B_{12} on HPLC, which hindered a quantification of both NCbas. Assuming that the ratio between the quantifiable NCba 2 and the NCba 1 that is not distinguishable from norpseudovitamin B_{12} on HPLC was constant

throughout all applied concentrations of MeBza with 1:2, the respective norpseudovitamin B_{12} curve in Fig. 1B would be highly similar to that of Bza treated cells.

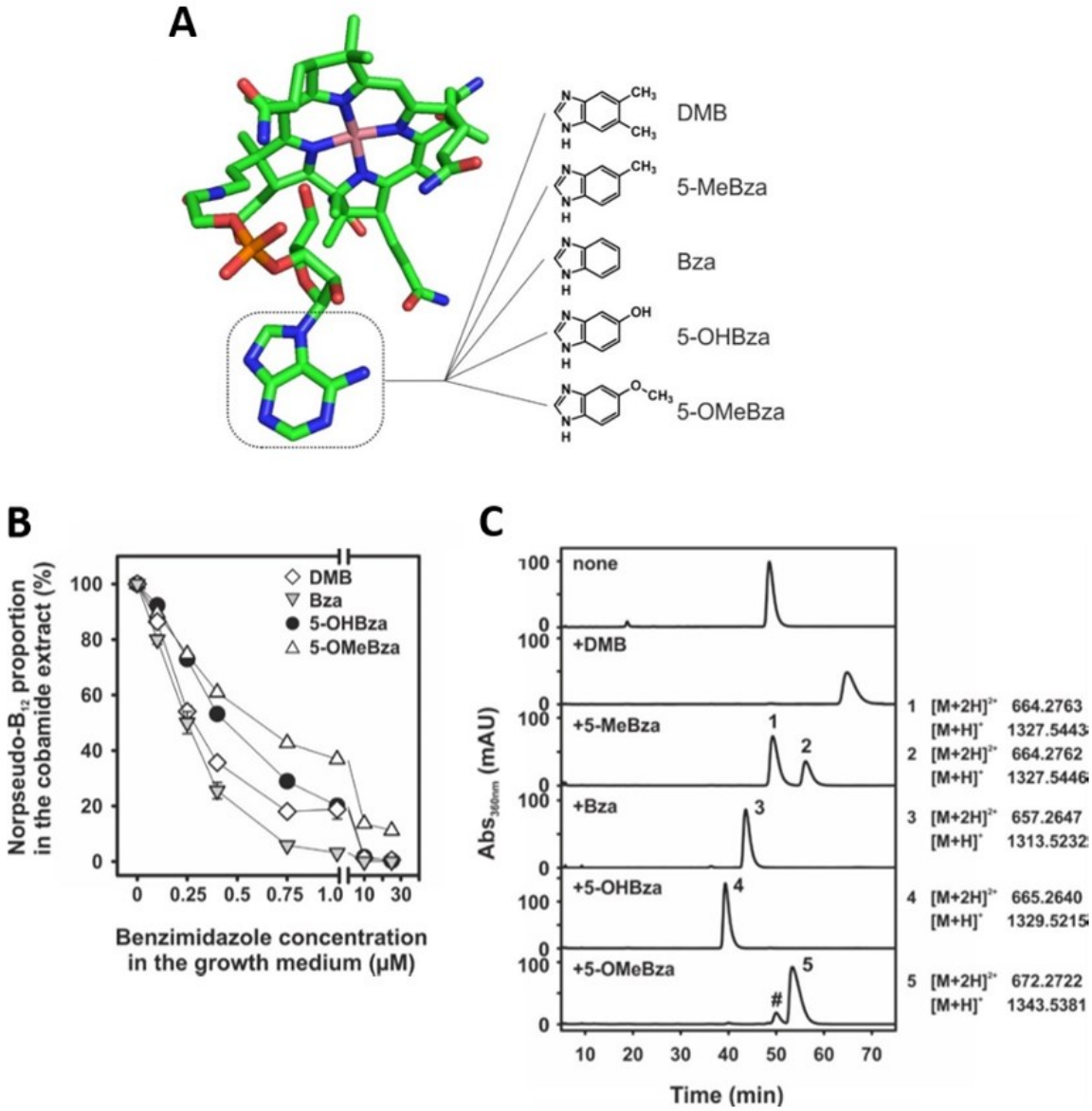

Fig. 1: Guided-NCba biosynthesis in *S. multivorans*. A) Norpseudo-B_{12} cofactor in the PceA-bound state. A frame marks the adeninyl-moiety in the nucleotide loop. The structures of the exogenous benzimidazoles, which were tested in the study, are depicted. B) Ratio between norpseudo-B_{12} and various benzimidazolyl-NCbas formed in the presence of different concentrations of exogenous benzimidazoles. C) HPLC-analysis and mass determination of NCbas extracted from *S. multivorans* cells cultivated in the presence of different benzimidazoles (25 µM, respectively). The hash marks the norpseudovitamin B_{12} in the Cba extract from cells treated with 5-OMeBza. Singly and doubly protonated ions were detected which were assigned to the respective NCbas: *m/z* 1327.5443 $[M+H]^+$ (calculated for $C_{61}H_{85}O_{14}N_{14}CoP$, 1327.5439, 0.745 ppm) and *m/z* 664.2763 $[M+2H]^{2+}$ (calculated for $C_{61}H_{86}O_{14}N_{14}CoP$, 664.2753, 1.492 ppm) for peak **1** and *m/z* 1327.5446 $[M+H]^+$ (calculated for $C_{61}H_{85}O_{14}N_{14}CoP$, 1327.5439, 0.873 ppm) and *m/z* 664.2762 $[M+2H]^{2+}$ (calculated for $C_{61}H_{86}O_{14}N_{14}CoP$, 664.2753, 1.266 ppm) for peak **2** of the sample derived from the 5-MeBza-

treated cells, *m/z* 1313.5232 $[M+H]^+$ (calculated for $C_{60}H_{83}O_{14}N_{14}CoP$, 1313.5238, -3.465 ppm) and *m/z* 657.2647 $[M+2H]^{2+}$ (calculated for $C_{60}H_{83}N_{14}O_{14}CoP$, 657.2675, -4.2 ppm) for the Bza-NCba **3**, *m/z* 1329.5215 $[M+H]^+$ (calculated for $C_{60}H_{83}O_{15}N_{14}CoP$, 1329.5232, -0.899 ppm) and *m/z* 665.2640 $[M+2H]^{2+}$ (calculated for $C_{60}H_{84}O_{15}N_{14}CoP$, 665.2650, -1.430 ppm) for the OHBza-NCba **4** and *m/z* 1343.5381 $[M+H]^+$ (calculated for $C_{61}H_{85}O_{15}N_{14}CoP$, 1343.5389, -0.168 ppm) and *m/z* 672.2722 $[M+2H]^{2+}$ (calculated for $C_{61}H_{86}O_{15}N_{14}CoP$, 672.2728, -0.887 ppm) for the OMeBza-NCba **5**. In the latter sample, ions for the residual norpseudo-B_{12} (peak **#**) were also detected at *m/z* 1330.5283 $[M+H]^+$ (calculated for $C_{58}H_{82}O_{14}N_{17}CoP$, 1330.5291, -0.607 ppm).

The analysis of purified NCbas formed in cells cultivated in the presence of 5-MeBza (Fig. 1C, NCba 1 and 2), 5-OHBza (NCba 4), and 5-OMeBza (NCba 5) by nuclear magnetic resonance (NMR) spectroscopy confirmed the assumption on the incorporation of the different benzimidazoles into the NCba structure (Fig. 2 and Fig. S1-26).

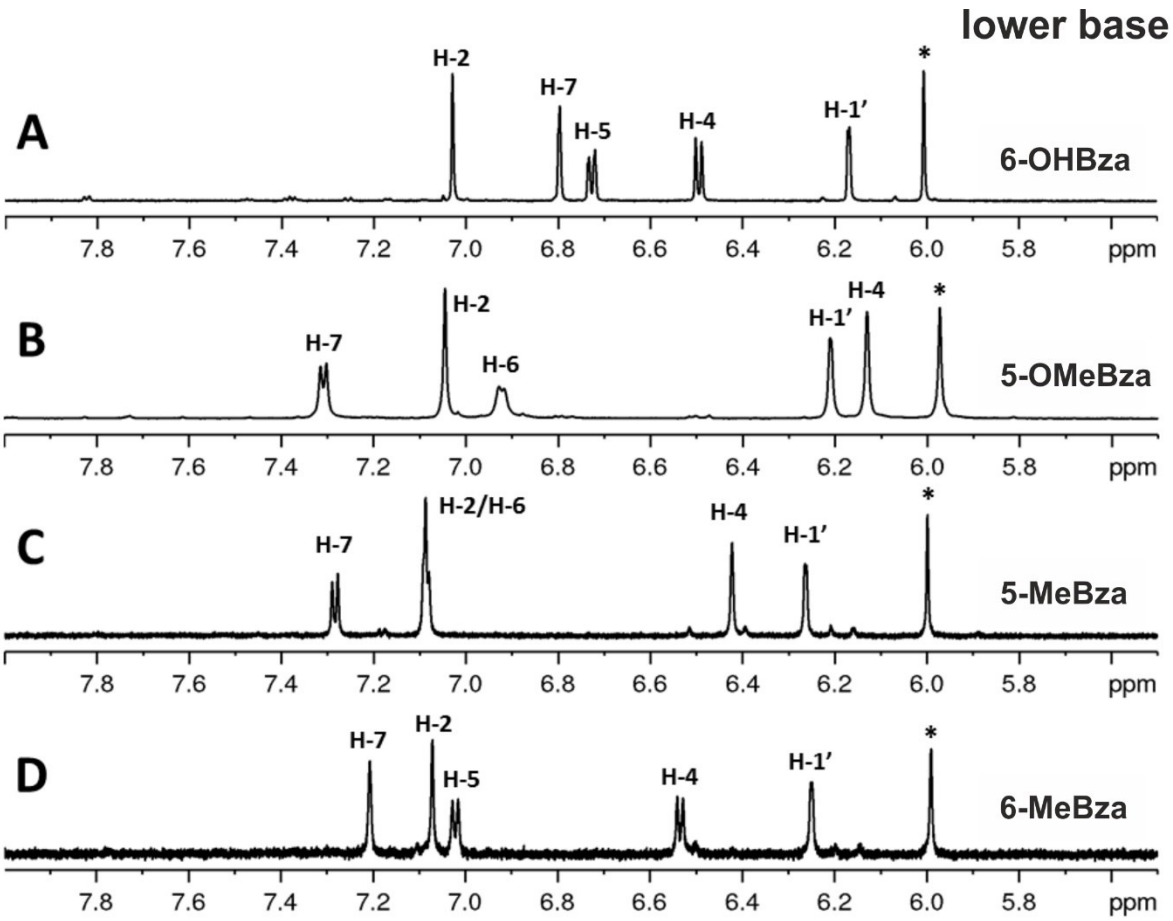

Fig. 2: Low field range of ^{1}H-NMR spectra of the isolated NCbas. (A) 6-OHBza-NCba, (B) 5-OMeBza-NCba, (C) 5-MeBza-NCba (NCba 1 in Fig. 1C), and (D) 6-MeBza-NCba (NCba 2 in Fig. 1C). The depicted sections show the signals for the respective benzimidazolyl moieties and the

signal for the anomeric position of the α-ribosyl unit. The signal marked with an asterisk belongs to position 10 of the corrin scaffold.

The determined chemical shifts obtained for the NCba 4 (Fig. 1C) from 5-OHBza-treated cells allowed for the identification of 6-OHBza-NCba (Fig. 2A) and in case of NCba 5 from 5-OMeBza-treated cells of 5-OMeBza-NCba (Fig. 2B). The data were in accordance with previously published results by Taga and coworkers (Crofts *et al.* 2014). In addition, the position of the substituent within the lower base structure was determined in both NCbas obtained from 5-MeBza-treated cells and the occurrence of two orientations of MeBza in the nucleotide loop was approved (Fig. 2 C, D). Hence, guided biosynthesis with 25 µM exogenous 5-MeBza results in the formation of 5-MeBza-NCba and 6-MeBza-NCba with a ratio of about 2:1 in *S. multivorans*.

Enzymatic activation of benzimidazoles. Some bacteria were previously shown to incorporate exogenous 5-OHBza or 5-OMeBza into cobamides with the respective substituent not just at C5, but also at C6 of the benzimidazole moiety of the B_{12} molecules (Crofts *et al.* 2014). This regioselectivity in Cba biosynthesis was previously dedicated to the function of the CobT enzyme, which activates the lower base precursors by the formation of α-ribotides (Fig. 3A). In order to test for the regioselectivity of the nicotinate mononucleotide/5,6-dimethylbenzimidazole phosphoribosyltransferase of *S. multivorans* (*Sm*CobT), the protein was heterologously produced, purified (Fig. 3B), and tested for the benzimidazole and adenine conversion (Fig. 3C). The conversion of the different benzimidazoles by *Sm*CobT in the enzymatic assay was monitored via HPLC coupled to photometric detection. The synthesis of the various α-ribotides from the different benzimidazoles tested in the assay was verified using UHPLC-ESI-HRMS. All tested benzimidazoles were activated by CobT *in vitro* (Fig. 3C). Due to an instability of the phosphate group of the ribotide products under the applied conditions, they were excluded from the determination of the conversion rates of CobT. Thus, the activity of the enzyme was monitored using the decrease of the signals for the free benzimidazole or adenine substrates on HPLC over time (Fig. 3C). The *Sm*CobT enzyme displayed a preference for 5-OHBza that was converted rapidly with the highest initial conversion rate (between 0-5 min) of 1.34 ± 0.05 nkat/mg. Adenine, the physiological lower ligand of the cobamide produced in *S. multivorans*, was activated with 1.24 ± 0.06 nkat/mg

CobT. A turnover rate, about 20 % of which was determined for 5-OHBza or adenine, was measured for DMB and 5-MeBza (0.29 ± 0.04 and 0.23 ± 0.04 nkat/mg protein, respectively). However, these low activities still allowed for an efficient use of DMB and 5-MeBza in the NCba biosynthesis *in vivo* (Fig. 1B-C).

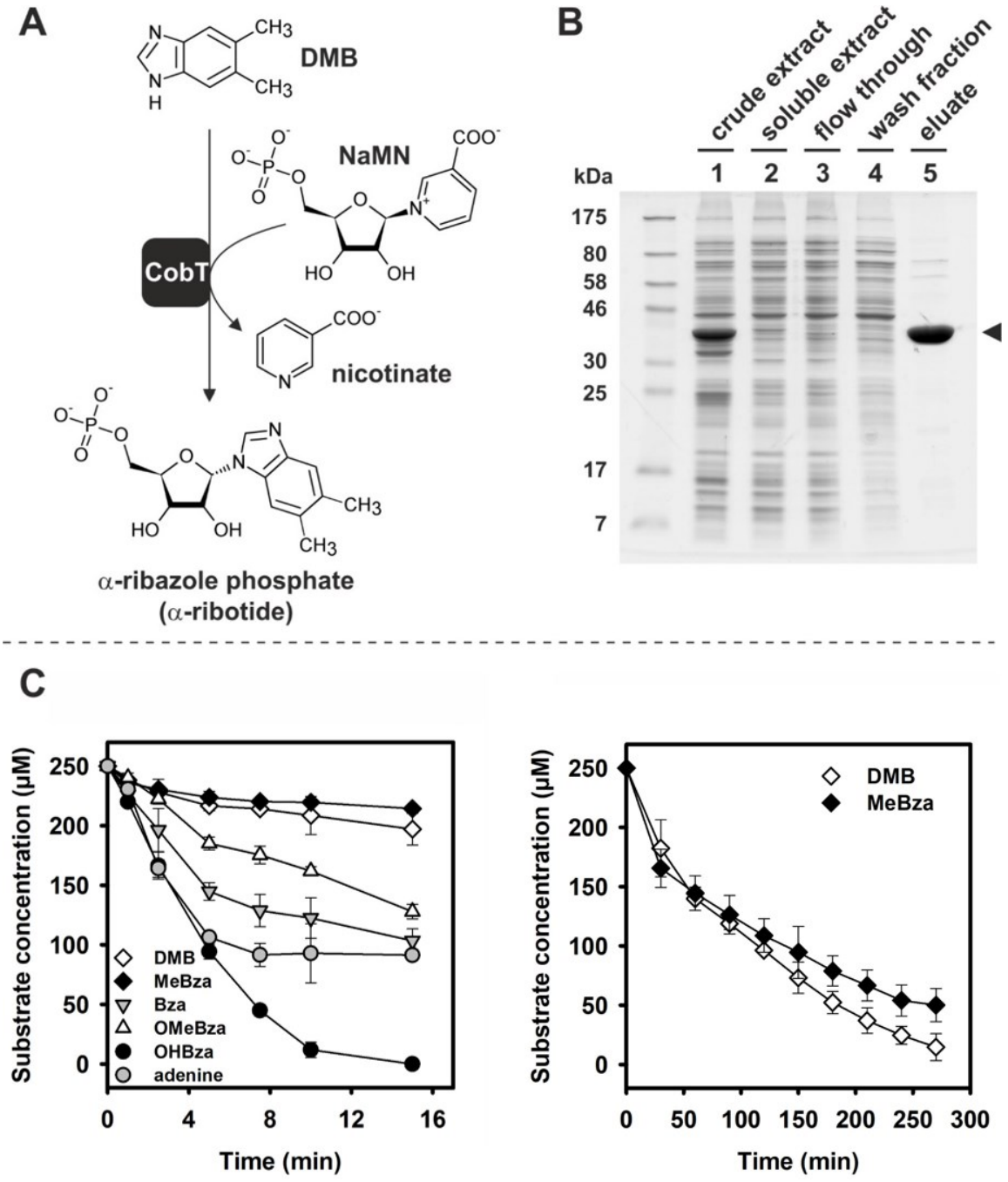

Fig. 3: Formation of α-ribotides by *Sm*CobT. A) Schematic presentation of the nicotinate mononucleotide/5,6-dimethylbenzimidazole phosphoribosyltransferase (CobT) reaction. B) SDS-PAGE displaying various fractions from the *Sm*CobT purification. C) *Sm*CobT-mediated conversion of different benzimidazoles and adenine monitored for a short term (left panel) or a long term (right panel). The data presented in part C are derived from two independent CobT

purifications and subsequent enzyme activity measurements. The standard deviations are given. NaMN = nicotinate mononucleotide. The following ions were detected that have been assigned to the monoisotopic masses of singly protonated benzimidazolyl and adeninyl α-ribotides along with the calculated mass and mass difference: *m/z* 359.1002 $[M+H]^+$ in case of the DMB α-ribotide (calculated for $C_{14}H_{20}O_7N_2P$, 359.1008, -0.1059 ppm), 345.0845 $[M+H]^+$ for the MeBza α-ribotide (calculated for $C_{13}H_{18}O_7N_2P$, 345.0852, -0.3747 ppm), 331.0689 $[M+H]^+$ for the Bza α-ribotide (calculated for $C_{12}H_{16}O_7N_2P$, 331.0695, -0.181 ppm), 347.0639 $[M+H]^+$ for the OHBza α-ribotide (calculated for $C_{12}H_{16}O_8N_2P$, 347.0644, 0.0404 ppm), 361.0795 $[M+H]^+$ for the OMeBza α-ribotide (calculated for $C_{13}H_{18}O_8N_2P$, 361.081, -0.1908 ppm), and 348.0705 $[M+H]^+$ for the adenine α-ribotide (calculated for $C_{10}H_{15}O_7N_5P$, 348.0709, 0.3429 ppm).

Surprisingly, the amount of free adenine did not further decrease after 10 min in the CobT assay and even increased slightly. This observation might be caused by an instability of the adenine α-ribotide under the applied conditions since the increase of free adenine in the assay was coherent with a decrease in the intensity of adenine α-ribotide on HPLC. However, the signal for the second product nicotinate increased steadily throughout the adenine assay excluding a potential backwards reaction of *Sm*CobT catalyzing free adenine.

HPLC analysis of the *Sm*CobT reaction mixture allowed for the separation of two α-ribotide product peaks in the case of the assay conducted with 5-OHBza (Fig. 4A) or 5-OMeBza (Fig. 4B). From the comparison of the absorbance spectra obtained for the two separated peaks in both cases with those reported by Taga and coworkers (Crofts *et al.* 2014), the synthesis of both isomers of the OHBza α-ribotide and the OMeBza α-ribotide became evident. A clear dominance of the 6-OHBza α-ribotide versus the 5-OHBza α-ribotide (9:1) was observed, while for the OMeBza-containing isomers a ratio of approximately 2:1 between the 5-OMeBza α-ribotide and the 6-OMeBza α-ribotide was detected. A separation of two peaks for the MeBza α-ribotide was not possible with the method applied (Fig. 4C). However, the NMR analysis of the purified MeBza α-ribotide fraction uncovered the presence of both isomers, the 5-MeBza α-ribotide and the 6-MeBza α-ribotide in a ratio of 2:1 (Fig. 4C, lowest panel).

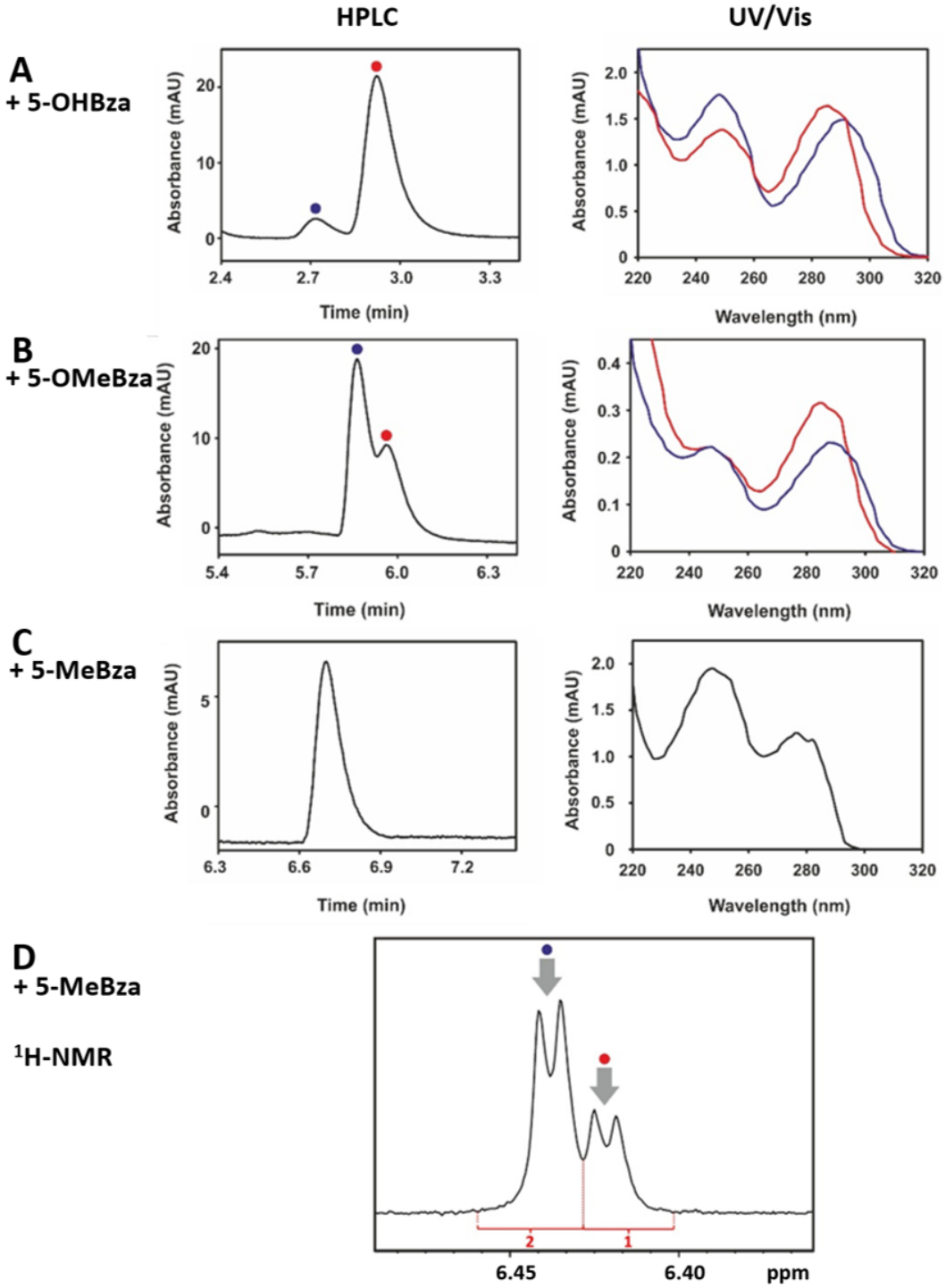

Fig. 4: Separation and identification of the α-ribotides formed in the *Sm*CobT assay. A) HPLC-elution profile of the 5-OHBza α-ribotide (*blue*) and the 6-OHBza α-ribotide (*red*) combined with the respective UV/Vis-absorbance spectra. The absorbance spectra were normalized at 260 nm. B) HPLC-elution profile of 5-OMeBza α-ribotide (*blue*) and 6-OMeBza α-ribotide (*red*) combined with the respective UV/Vis-absorbance spectra. The absorbance spectra were normalized at 250 nm. C) HPLC-elution profile of the MeBza α-ribotide combined with the UV/Vis-spectrum of the purified fraction and with a ^{1}H-NMR spectrum showing the signals of the anomeric position of the α-ribotide moiety (D). According to the ratio of integrals (indicated by the red brackets) a 2:1 mixture of 5-MeBza- (*blue*) and 6-MeBza-α-ribotide (*red*) was present in the sample.

Effect of singly substituted benzimidazoles on PceA formation. The exchange of the adeninyl moiety in the norpseudo-B_{12} cofactor by DMB caused a negative effect on the PCE-dependent growth and the formation of catalytically active PceA in *S. multivorans* (Keller *et al.* 2014). Up to date, no other benzimidazoles were tested in this respect. Thus, 5-MeBza, Bza, 5-OHBza, or 5-OMeBza (25 µM, respectively) were added to cultures of *S. multivorans* that contained pyruvate and PCE as substrates. None of the benzimidazoles affected the PCE-dependent growth of the organism except DMB (Fig. 5A). However, in order to exclude any impact on the PceA function, the enzyme activity was tested in crude extracts of the benzimidazole amended cells (Fig. 5B). For this purpose the cells were harvested in the late exponential growth phase, *i.e.*, the protein concentration was 60-80 µg/ml, which was reached after approx. 16 hours of cultivation under the applied conditions. In the case of the DMB-containing cultures, the same growth phase was reached after approx. 80-90 hours of cultivation (Fig. 5A). Crude extracts were prepared and the specific PceA activity was measured in a photometric assay using reduced methyl viologen as artificial electron donor. The activity determined in cells treated with either Bza, 5-OHBza, or 5-OMeBza were similar to the PceA activity monitored in the non-treated *S. multivorans* cells (Fig. 5B). In contrast, the presence of 5-MeBza caused a substantial reduction in the enzyme activity (about 50%). This reduction was not as high as in DMB-treated cells (about 90%) and appeared to be still sufficient for nearly unaffected growth with PCE (Fig. 5A).

The reduction in PceA activity in cells cultivated in the presence of 5-MeBza might be caused by a retardation in prePceA maturation and processing as it was observed for DMB before (Keller *et al.* 2014). Thus, the presence of both forms of the enzyme were analyzed in crude extracts separated on an SDS-PAGE and applied to an immunoblot developed with PceA-specific antibodies (Fig. 5B). In comparison to the crude extract of non-treated cells, which displayed an intense band for the mature PceA enzyme (predicted molecular mass: 53.3 kDa) and only a thin band for the Tat (twin arginine translocation) signal peptide-containing prePceA (predicted molecular mass: 57.1 kDa), the amount of prePceA was increased in 5-MeBza-treated cells. The total amount of the enzyme and its processing was not affected in cells cultivated in the presence of Bza, 5-OHBza, and 5-OMeBza as detected by the immunoblot analysis.

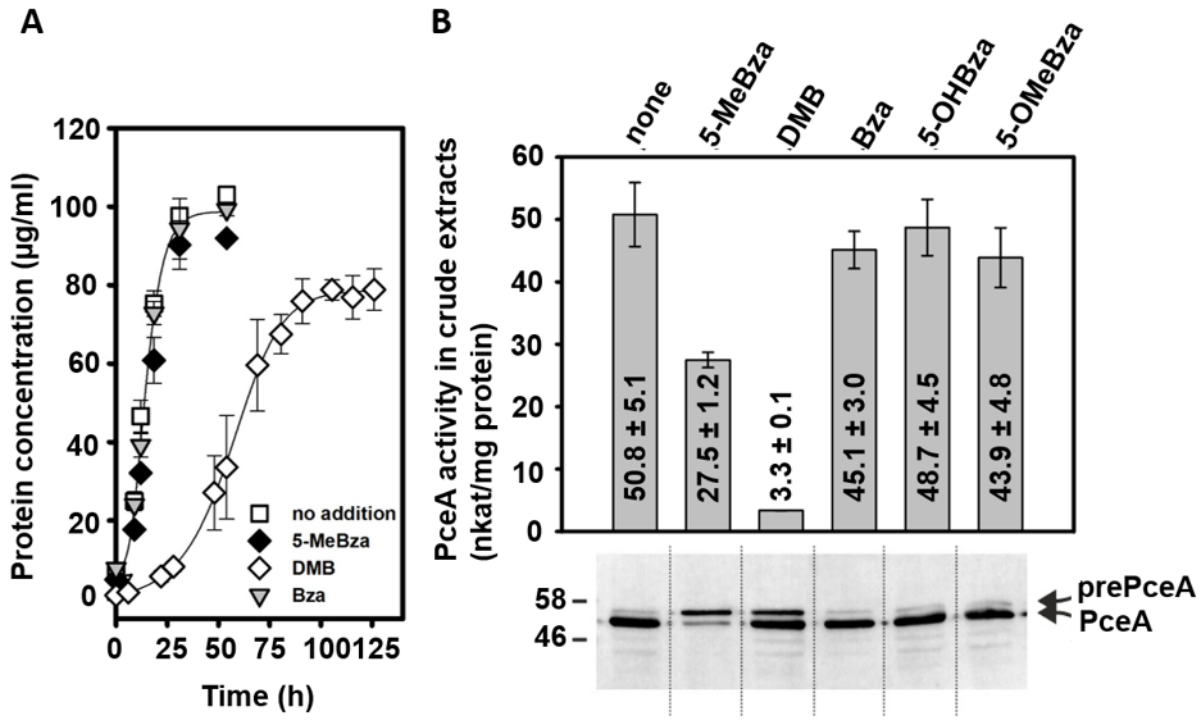

Fig. 5: A) Growth of the third subculture of *S. multivorans* on pyruvate/PCE medium amended with 25 µM of DMB, 5-MeBza, Bza, or without any benzimidazole. The 5-OHBza and 5-OMeBza curves were excluded due to the same growth behavior like with Bza. The standard deviation of two cultures is given. B) PceA activity and protein level in crude extracts derived from cells grown with PCE in the presence of different benzimidazoles (25 µM). For the immunological analysis 5 µg of protein was applied to each lane. The data for the PceA activity were obtained from three different cultures. The standard deviation is given. prePceA: precursor with the N-terminal Tat signal peptide, PceA: processed form without the Tat signal peptide.

Binding of benzimidazolyl-NCbas to PceA. In a previous study, DMB was already shown to hamper the PceA activity by becoming the lower base of the NCba produced by the organism (Keller *et al.* 2014). A quantification of the incorporation efficiency of the DMB-NCba into PceA was difficult, since a sufficient amount of pure PceA enzyme was not obtained from such DMB-treated cells. These reduced PceA amounts and the negative effect on the maturation of the PceA precursor under these conditions indicated an incompatibility between the DMB-NCba and the enzyme. The alternative doubly substituted benzimidazoles such 5,6-dinitrobenzimidazole (DNO_2Bza) and 5,6-dimethoxybenzimidazole (DOMeBza) were tested in this study (data not shown). In case of DNO_2Bza (25 µM in the growth medium) a reduction of about 80 % in the PceA activity in crude extracts was observed, comparable to what was found before with DMB. While in DNO_2Bza-treated cells the norpseudo-B_{12} cofactor was nearly

completely replaced, exogenous DOMeBza (25 µM) replaced less than 5 % of the adenine moieties in the norcobamide fraction, which hindered the evaluation of its effect on the PceA function. Hence, DOMeBza was the first tested benzimidazole derivative that displayed a poor guided biosynthesis efficiency in *S. multivorans*. In the *Sm*CobT assay both, DNO_2Bza and DOMeBza, were substantially converted to the respective α-ribotide (data not shown).

In contrast to the effects observed with the doubly substituted benzimidazoles, the analysis of the PceA activity in cells treated with 5-OHBza or 5-OMeBza showed no negative effect (Fig. 5). This finding indicated an efficient utilization of both NCbas, the 6-OHBza-NCba and the 5-OMeBza-NCba, as cofactor of the PceA enzyme. For unravelling similarities or differences in the binding mode of these cofactors and for obtaining some indications for the exclusion of the DMB-NCba, the PceA enzyme was purified from the respective type of cells, crystallized, and subjected to structural analysis. Cells cultivated in the presence of 5-MeBza were also included in the analysis. However, crystals of PceA suitable for X-ray scattering and structural analysis were only gained for the enzyme purified from cells treated with 5-OHBza or 5-OMeBza. Extraction of the norcobamides from purified PceA uncovered a 100 % saturation of the enzyme (1 mol cofactor per 1 mol PceA) in all cases, including enzyme extracted from 5-MeBza-treated cells, and the elution profiles of the extracted cofactors obtained via HPLC separation (Fig. 6) mirrored the patterns observed earlier for the NCbas extracted from whole cells (Fig. 1C). The inability to dissolve the PceA structure from MeBza grown cells might indicate an inhomogeneity of the sample and the presence of misfolded PceA. This assumption was supported by the fact that the specific activity of PceA purified from cells cultivated on pyruvate/fumarate medium in the presence of 5-MeBza was about 50 % lower compared to the PceA purified from non-treated cells (687 vs. 1312 nkat/mg, respectively).

In both known RDase structures (Bommer *et al.* 2014, Payne *et al.* 2015), the Cba cofactor is deeply buried within the protein. Access to the upper β-face of the corrin ring within the substrate binding pocket of PceA is limited by the protein structure. The nucleotide loop of the adeninyl-NCba (norpseudo-B_{12}) in PceA is positioned in the 'base-off' conformation (Fig. 7A). The unusual curled conformation of the tail is stabilized by intramolecular interactions - hydrogen bonds between O5 of the ribosyl moiety (for numbering of the atoms in norpseudo-B_{12} see Kräutler *et al.* 2003), the linker amide, and the carboxamide side chain connected to ring C of the corrin core (Fig. 7B) (Bommer *et al.* 2014).

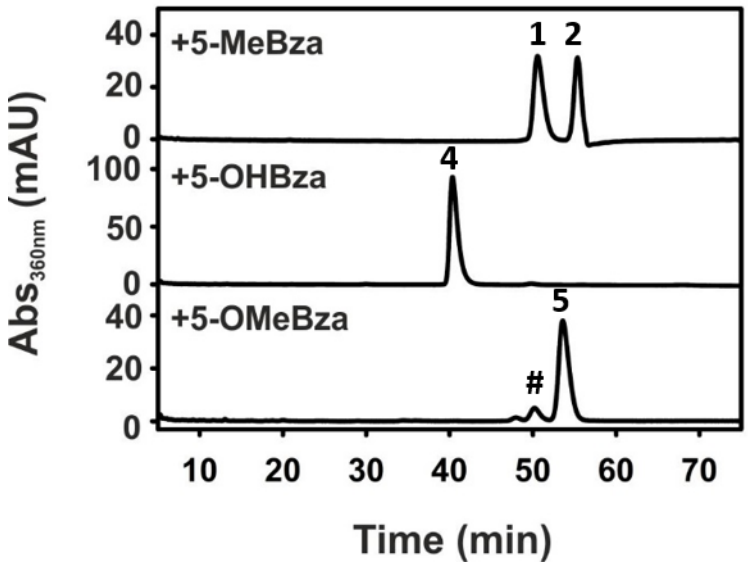

Fig. 6: HPLC-elution profiles of NCbas extracted from PceA purified from *S. multivorans* cultivated in the presence of either 5-MeBza, 5-OHBza, or 5-OMeBza. The numbers refer to the NCbas from Fig. 1C.

These stabilizing interactions are conserved in both, the PceA equipped either with the 6-OHBza-NCba or the 5-OMeBza-NCba. Another intramolecular hydrogen bond connects the amino group of the adenine and O2 of the ribosyl moiety only in the adeninyl-NCba (Fig. 7B). The tight binding of the phosphate and sugar moieties in all PceA structures restricted the movement of the base to a rotation of the bond connecting base and sugar. The same rotation in turn is restricted by the protein surroundings. The base is positioned between the Cba-binding core of PceA (*purple* in figure 7) and a loop from the region binding the iron-sulfur clusters (Bommer *et al.* 2014). Lys362 from this loop is within hydrogen-bonding distance of both, the base and the phosphate. Gln364 reaches across the base and hydrogen bonds to O2 of the sugar. Thus, intramolecular and protein interactions effectively lock the position of the base and this is reflected by the conserved position observed for both benzimidazole-containing NCbas (Fig. 7C and D). In PceA but not in the structure of the non-respiratory RDase *Np*RdhA from *Nitratireductor pacificus* (Payne *et al.* 2015), a solvent channel reaches from the protein surface to the far end of the base and the amino group and N1 of the adeninyl base is in hydrogen-bonding distance to water molecules (Fig. 7B). The three different substituents on the various bases, namely the amino group of the adeninyl moiety (Fig. 7B), the hydroxyl group of the 6-OHBza moiety (Fig. 7C), and the methoxy group of the 5-OMeBza moiety (Fig. 7D) are accommodated by a change in the water structure rather than change in the position of the base or a reorientation of surrounding side chains.

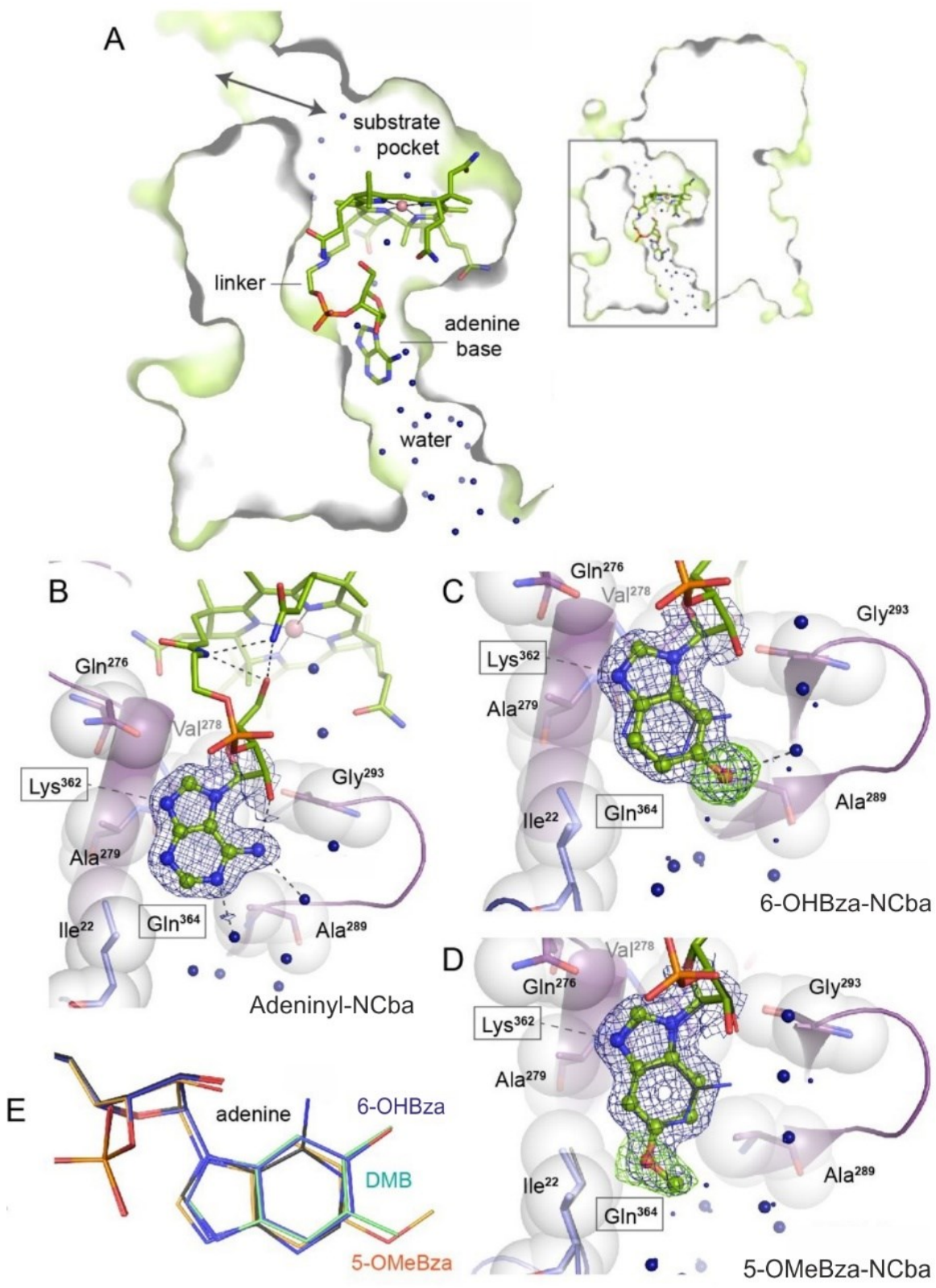

Fig. 7: NCba cofactor-binding in PceA. A+B) Slice through a monomer of PceA showing the norpseudo-B_{12} cofactor. The adenine base (ball and stick in B) is sandwiched between the B_{12}-binding core (residues 139-163 and 216-323, behind the view, *purple*) and a loop from the iron-sulfur cluster binding unit (residues 324-394, in front of the view and thus not shown but Lys362 and Gln364 are indicated as boxes). Lys362 is within hydrogen bonding distance of the imidazole moiety in all three bases (*dashed lines*). C) When 6-OHBza is substituted for adenine,

only the base is shifted slightly (thin lines represent the position of norpseudo-B_{12}). Greater change was observed for the water structure (small dots denote water positions in norpseudo-B_{12} bound PceA). Electron density maps are shown for the base only (*blue* 1 σ 2Fo-Fc and *green* 2.5 σ Fo-Fc difference maps when the benzimidazole substituents were omitted). D) The same is shown for the 5-OMeBza-NCba. E) Overlay of the bases in B-D and a hypothetical positioning of DMB, which was overlayed to 6-OHBza.

The closest interactions between the new substituents were seen between Ile22 of the N-terminal loop region and the 5-OMeBza moiety (3.5 Å) and between Ala289 and the 6-OHBza moiety (3 Å). A substitution at a position other than C5 or C6 may not be possible without a change in the protein scaffold. Given the positioning of the 6-OHBza- and the 5-OMeBza-moieties in PceA, no clash with the protein would be expected with a hypothetical MeBza or DMB as lower base (Fig. 7E). Hence, the static, folded structure cannot fully explain the apparent lack of functionality of the MeBza-NCba and the DMB-NCba. The unique feature of PceA is the solvent accessible and water-bound base. Such solvation might play a role in cofactor acquisition and correct functional folding of both PceA enzyme and cofactor.

DISCUSSION

The lower base of the cobamide cofactors in most Cba producing prokaryotes is either a benzimidazole or a purine derivative (Renz 1999, Cheong *et al.* 2001, Taga and Walker 2008). The main function of the lower base in enzymes harboring a 'base-off' cobamide cofactor is a tight binding to the protein and thus stabilizing the correct position of the cobamide in the enzyme (Drennan *et al*. 1994, Mancia *et al.* 1996, Reitzer *et al.* 1999, Bommer *et al.* 2014, Payne *et al.* 2015). The diversity of benzimidazoles and purines might interfere with the binding of the cofactor to Cba-dependent enzymes such as RDases. Both structural analyzed dehalogenases, PceA of *S. multivorans* and NpRdhA from *Nitratireductor pacificus* pht-3B were shown to bind the cofactor with a network of hydrogen bonds in the 'base-off' mode deeply inside the protein (Bommer *et al.* 2014, Payne *et al.* 2015). However, the molecular basis for the diversity in the Cba structure and for the observed incompatibilities between Cba cofactors and RDases, reported for selected reductively dehalogenating bacteria, was not investigated so far. Furthermore, the regioselectivity of the various CobT enzymes with singly substituted benzimidazoles as previously observed for the respective enzymes of

Sinorhizobium meliloti, Salmonella enterica, and *Veillonella parvula*, is mostly unknown in these organisms (Crofts *et al.* 2014). An in depth analysis of these specificities on molecular level might help to understand the physiological limitations selected OHRB are underlying in this respect. In the study presented here, the NCba-containing PceA enzyme of *S. multivorans* was investigated for the effect of exogenously applied benzimidazoles on the RDase formation and function. The structural analysis of PceA equipped with either the purine or benzimidazole-containing NCbas revealed no difference in binding of the cofactor in general and the lower base in particular. Actually, the positioning of the lower base was found to be nearly identical. With these data *Sm*PceA is the only cobamide-dependent enzyme, whose structure was determined with purine and benzimidazole-containing cobamide cofactors. However, from the tested benzimidazoles only Bza, 5-OHBza, and 5-OMeBza, exogenously applied, revealed no negative effects on the PCE-metabolism and the PceA activity and hence a compatibility of the respective norcobamides and the RDase. In the presence of methylated benzimidazolyl NCbas the activity of the enzyme was affected and in case of DMB, the respective NCba was most probably excluded from the incorporation into PceA. In the case of MeBza, an incorporation at all did not appear to be hindered, since both NCba isomers, the 5-MeBza- and the 6-MeBza-containing variant, were detected in the enzyme. Nevertheless, the enzyme activity of the pool of PceA molecules containing both isomers was reduced and the difficulties observed in the crystallization of purified PceA indicated an inhomogeneity of the sample. Moreover, the crystallization of 5- and 6-MeBza NCba-containing PceA resulted in a different crystal packing in which the base could not be fully resolved, preventing a detailed structural analysis of the cobamide binding and conformation situation in this case. Since OHBza or OMeBza, incorporated as lower bases in 6-OHBza-NCba or 5-OMeBza-NCba, were functional in the enzyme, it is hard to conclude on the specific isomer of MeBza-NCba that causes PceA malfunction. No structural clash between the substituents at the benzimidazole moiety and the protein environment were found in the structure. Hence, it has to be assumed that the lower base substitution pattern might interfere with the PceA folding process rather than with the dimensions of the binding pocket present in the fully-folded enzyme. Whether the cofactor has to attain a specific conformation prior to the incorporation into the enzyme and whether this might be differentially affected in the various benzimidazolyl NCbas tested here remains elusive. The hydrophobic methyl groups in DMB and MeBza could interfere with the incorporation process. The lower base of the NCba cofactor in PceA is placed in a solvent-

flooded cavity, which might be incompatible with the hydrophobic character of the methylated benzimidazoles. The DMB of the Cba cofactor in *Np*RdhA is protected from solvent (Payne *et al.* 2015). It was observed earlier that the structure of the lower base influences the ratio between the 'base-off' and the 'base-on' form of Cbas in solution. Kräutler and coworkers showed the adenine-containing pseudocoenzyme B_{12} having a higher tendency to occur in 'base-off' conformation in solution compared to the DMB-containing coenzyme B_{12} (Fieber *et al.* 2002). In addition, the internal hydrogen bridge linkage between the adenine and the ribose moiety of the nucleotide loop in norpseudo-B_{12} might be supportive for obtaining the correct topology and for a successful incorporation into PceA. Even between structural isomers, the tendency to obtain the 'base-off' conformation differs. Taga and coworkers reported a lower pK_a value for 5-OMeBza-Cba in comparison to 6-OMeBza-Cba (Crofts *et al.* 2014). However, a systematic analysis of the impact of all potential lower bases on the constitution of the cofactor under physiological conditions is not available, which make predictions about their influence on having the cofactor in the 'base-off' mode prior to incorporation into PceA difficult. A cooperative folding of NCba cofactor and the PceA protein cannot be excluded that lead to the final constitution of the enzyme-cofactor complex. Not all RDases seem to bind the Cba cofactor in the 'base-off' mode. As recently reported for the reconstituted vinyl chloride reductive dehalogenase VcrA of *D. mccartyi* strain VS, the DMB-containing Cba cofactor showed the lower ligand bound to the cobalt in the enzyme-bound state (Parthasarathy *et al.* 2015). Furthermore, other RDases showed different preferences for the type of lower base in the Cba cofactor (Yi *et al.* 2012, Yan *et al.* 2012, Yan *et al.* 2013, Yan *et al.*, 2015), which implies that a general rule for the cofactor compatibility of RDases does not exist. The role of chaperones in the Cba cofactor incorporation was not investigated so far. Specific chaperones, such as RdhT homologs (Morita *et al.* 2009, Maillard *et al.* 2011) are not found in *S. multivorans* and have not been identified in *D. mccartyi*. Banerjee and coworkers reported the transfer of the Cba cofactor to methylmalonyl-CoA mutase to be mediated by the adenosyltransferase (ATR) (Padovani *et al.* 2008). An ATR homolog is not present in *S. multivorans*, most probably since the NCba cofactor of PceA is catalytically active without the adenosyl moiety as upper ligand of the cobalt ion.

The results obtained in this study show the potential diversity of Cbas that might be formed within a single OHRB when lower base precursors are provided. The lower ligand activating enzyme *Sm*CobT confirmed previous reports on the broad substrate range of CobT homologs

from other organisms (Cheong *et al.* 2001, Crofts *et al.* 2013, Crofts *et al.* 2014). The low activation rates of CobT observed for the methylated benzimidazoles 5-MeBza and DMB might be interpreted as an adaptation to the incompatibility of PceA with the resulting NCbas. Whether the dominance in the formation of 6-OHBza-NCba or 5-OMeBza-NCba reflects an adaptation to the needs of the PceA enzyme remains speculative. From the mixture of α-ribotides formed by *Sm*CobT in case of 5-OHBza or 5-OMeBza as substrates almost exclusively one isomer is found in the final NCba fraction. This result points towards the existence of another selective step within the nucleotide loop assembly pathway that discriminates between the two CobT product isomers for incorporation in the final Cba. A regioselectivity of the CobS enzyme (cobalamin 5'-phosphate synthase, EC 2.7.8.26), which fuses the α-ribotide and cobinamide-GDP (Escalante-Semerena 2007) might exist. Overall, making predictions for the outcome of the Cba biosynthetic pathway guided with singly substituted benzimidazoles in other OHRB is difficult, since the amino acids of the CobT's C-terminus, proposed to be involved in defining the fate of the substrate (Cheong *et al.* 2001), have little or no similarity. Overall, for elucidating the cofactor requirements of a certain OHRB an analysis of its capacity in incorporating lower bases into Cba cofactors might be helpful. However, it seems that it does not necessarily reflect the needs of a specific RDase enzyme in the organism.

EXPERIMENTAL PROCEDURES

Cultivation of bacteria. *Sulfurospirillum multivorans* (DSMZ 12446) was cultivated as described previously in Keller, 2014. Anoxic and sterile stock solutions of the different benzimidazoles (up to 4 mM) were prepared in ultrapure water (UPW) and used for the amendment of the media prior to the inoculation. All benzimidazole derivatives were purchased from Sigma-Aldrich (Munich, Germany), except for 5-OHBza that was delivered by Combi-Blocks, Inc. (San Diego, CA, USA) and DNO_2Bza that was purchased form Santa Cruz Biotechnology (Dallas, TX, USA). Cells of *S. multivorans* were adapted to the different cultivation conditions in two subsequent pre-cultures prior to the cultivation of cells for growth monitoring, PceA activity measurements, or cobamide extraction. In every case, the first pre-culture was inoculated from a pyruvate/PCE-grown culture of *S. multivorans* routinely prepared for strain maintenance.

Determination of the PceA activity, immunoblot, cobamide extraction and HPLC analysis. The production of cell free extracts and the determination of the PceA enzyme activity in crude extracts from *S. multivorans* cells was performed as described previously (Keller *et al.* 2014). This also applies to immunological detection of PceA in cell free extracts. The cobamide extraction and analysis was conducted in accordance to the protocol published earlier (Keller *et al.* 2014). The cells were cultivated in 1 liter pyruvate/fumarate-containing medium and harvested when the optical density (OD_{578}) reached 0.55. The mobile phases used for separation of the cobamides via high-performance liquid chromatography (HPLC) were 12 % methanol/0.2 % acetic acid (solvent A) and 99.8 % methanol/0.2 % acetic acid (solvent B). The flow rate was 0.5 ml/min and the separation was performed isocratically at 30 °C. The 6-OHBza norcobamide was purified additionally via solid phase extraction on a CHROMABOND® HR-X column (3 ml, 200 mg; Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

UHPLC-ESI-HRMS and measurements. For UHPLC, an Ultimate 3000 series RSLC (Dionex, Sunnyvale, CA, USA) was used applying an Acclaim C18 column (150 × 2.1 mm, 2.2 µm particles with 120 Å pore diameter, Dionex, Sunnyvale, CA, USA) with a flow rate of 300 µl min^{-1} in a binary solvent system of water (Solvent A) and acetonitrile (hypergrade for LC MS, Merck, Darmstadt Germany) (Solvent B), both containing 0.1 % (v/v) formic acid (eluent additive for LC-MS, Sigma Aldrich, Munich, Germany). Sample volumes were loaded onto the column and eluted by using a gradient as follows: linear increase from 0 % B to 100 % B within 15 min – 100 % B constant for 5 min – equilibration time at 0 % B for 5 min. This system was coupled to a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Ionization was accomplished using Electrospray Ionization (ESI). ESI source parameters were set to 4 kV for spray voltage, 35 V for transfer capillary voltage at a capillary temperature 275 °C. The samples were measured in positive ion mode in the mass range of m/z 100 to 2000 using 30,000 m/Δm resolving power in the Orbitrap mass analyzer. Data was evaluated and interpreted using XCALIBUR software (Thermo Fisher Scientific, Waltham, MA, USA).

NMR measurements. NMR spectra (^{1}H NMR, ^{1}H-^{1}H COSY, ^{1}H-^{1}H ROESY, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC) were recorded on a Bruker Avance III HD 700 spectrometer, equipped with a cryoplatform and a 1.7 mm TCI microcryoprobe (Bruker Biospin GmbH, Rheinstetten,

Germany). NMR tubes of 1.7 mm outer diameter were used for all measurements. All NMR spectra were recorded using D_2O as a solvent. Accurate tuning of the spectrometer frequencies (700.45 MHz for ^{1}H and 176.14 MHz for ^{13}C) was accomplished prior to the experiments. Chemical shifts were left uncorrected. Data acquisition and processing were accomplished using TopSpin ver3.2. (Bruker Biospin). Standard pulse programs as implemented in TopSpin were used for data acquisition.

Production, purification, and enzymatic assay of *Sm*CobT. For the heterologous expression of the *Sm*CobT enzyme in *E. coli* BL21 (DE3) the *cobT* gene sequence was amplified via PCR using genomic DNA of *S. multivorans* as template. By the use of the forward primer 5'-GCTGCCATGGATCTATTAGAAGAGAC-3' and the reverse primer 5'-TAATCTCGAGTACCTCTGGGTAGACACG-3' an *Nco*I and *Xho*I restriction site were introduced into the PCR product, which allowed for the ligation into the equally cut pASK-IBA63C-plus plasmid (IBA, Göttingen, Germany). The *cobT* sequence was verified by DNA sequencing. The *E. coli* strain harboring the expression plasmid was cultivated on LB medium at 37°C in the presence of 100 µg/ml ampicillin. When an $OD_{550\,nm}$ of 0.5 was reached the cells were induced with anhydrotetracycline (200 ng/ml) and the cells were harvested by centrifugation 3 h after induction. The *Sm*CobT enzyme was purified using the Streptactin Superflow column material (IBA, Göttingen, Germany) according to the manufacturer's instructions. The cells were disrupted by using a French Press at 1,000 Psi (French Pressure Cell Press, Thermo Fisher Scientific, Germany) and the resulting extract was subjected to ultracentrifugation (100,000 × g, 45 min, 4 °C; L8-M Ultracentrifuge, Rotor Ti70; Beckman Coulter, Krefeld, Germany). The supernatant (soluble extract) was used as starting material for the column chromatography. The eluate containing the purified enzyme was concentrated via ultrafiltration in a Vivaspin 6 (30 K) centrifugal concentrator (Sartorius, Göttingen, Germany). The *Sm*CobT activity was measured in an assay established by Taga and coworkers (Hazra *et al.* 2013). The reaction mixture contained 2 mM nicotinate mononucleotide (NaMN; Sigma Aldrich, Munich, Germany), 10 mM $MgCl_2$, 0.25 mM of the respective benzimidazole derivative, and 10 µM purified CobT-Strep in 50 mM Tris-HCl pH 7.5. The reactions were conducted at 24 °C and shaken at 300 rpm. The reaction was stopped by the addition of formic acid to a final concentration of 4 % and boiling of the sample at 100 °C for 1 min. Afterwards the denatured protein was precipitated by the addition of NaOH (0.75 %), storage on ice, and

subsequent centrifugation. The analysis of the substrates and products of the assay was performed via reverse phase HPLC (Smartline System, Knauer GmbH, Berlin, Germany) coupled to a diode array detector. The stationary phase was a Chromolith Performance RP-18e column, (100-4.6 mm; Merck, Darmstadt, Germany). The mobile phases were 10 mM ammonium acetate pH 6.5 (solvent A) and methanol (solvent B). The flow rate was 1 ml/min. The HPLC program applied here was identical to the procedure described in Hazra *et al.* 2013. The *Sm*CobT enzyme activity was calculated based on the data obtained for the reduction in the signal of the free bases (benzimidazoles or adenine) within the first 5 min of the reaction.

Crystallization of PceA and structural analysis. Crystallization and flash cooling of crystals were performed under anoxic conditions in a glove box (model B; COY Laboratory Products, Grass Lake, MI, USA) under an atmosphere of 95 % N_2/5 % H_2 and less than 10 ppm oxygen. Crystals were grown by the sitting drop vapor diffusion method at room temperature. 1 µL of 5-15 mg ml^{-1} PceA in 30 mM Tris-HCl, pH 7.5, 5 mM TCEP was mixed with 1 µl of crystallization solution containing 12-17 % (w/v) PEG 3350 and 0.2 M sodium malonate, 2 % benzamidine-HCl and 50 mM Tris-HCl, pH 7.5. Crystals were flash cooled in liquid nitrogen after protection in the crystallization solution supplemented with 20 % (v/v) glycerol and 25 % (final w/v) PEG 3350. Once plunged into liquid N_2, crystals were removed from the anoxic atmosphere, from thereon stored, and handled under liquid N_2.

Diffraction data for PceA equipped with 5-OMeBza norcobamide were collected on BL14.1 operated by the Helmholtz-Zentrum Berlin (HZB, Germany) at the BESSY II electron storage ring (Berlin-Adlershof, Germany) (Mueller *et al.* 2012). Data for PceA equipped with 6-OHBza norcobamide were collected at beamline P11 at PETRA-III (DESY, Hamburg, Germany) (Meents *et al.* 2013, Burkhardt *et al.* 2016). Data were indexed and integrated with the XDS package (Kabsch 2010) and XDSAPP (Krug *et al.* 2012). Restraints for the base were generated by the Grade Server v1.001 and used to modify the vitamin B_{12} restraint file posted by Oliver Smart (Global Phasing Ltd., Cambridge, UK). Models were fitted in COOT (Emsley *et al.* 2010), refined with phenix.refine (Afonine *et al.* 2012) and validated with Molprobity (Chen *et al.* 2010). Data collection and refinement statistics are summarized in the Table S1. Model coordinates and structure factors for PceA equipped with different norcobamides have been deposited at the protein data bank (PDB) under accessions numbers 5OBP (PceA with 6-OHBza norcobamide cofactor) and 5OBI (PceA with 5-OMeBza norcobamide cofactor).

ACKNOWLEDGEMENTS

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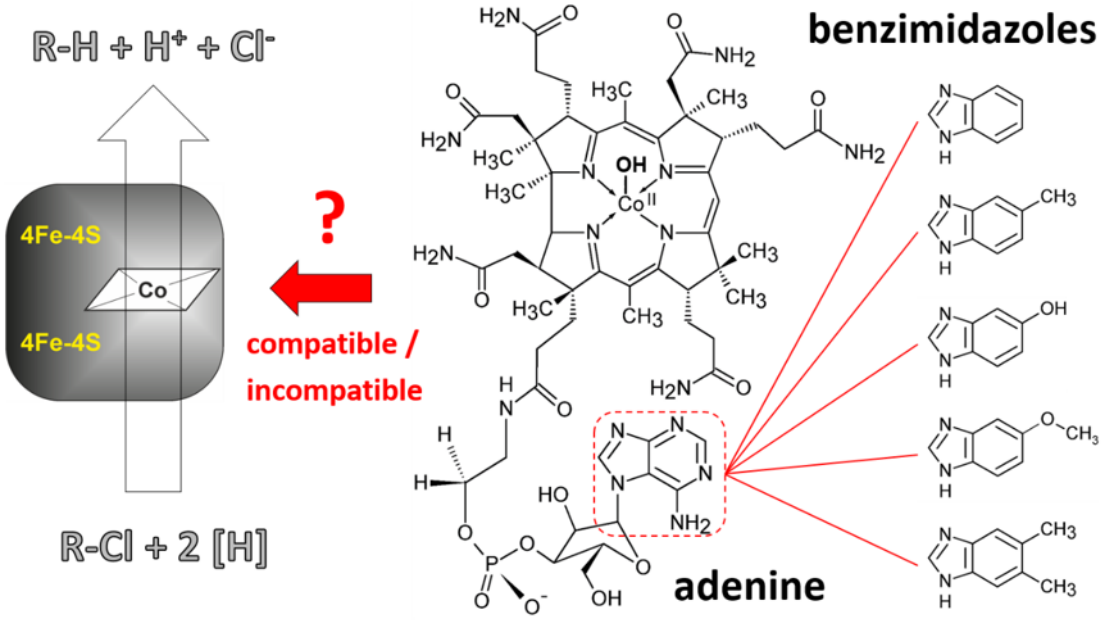
AUTHOR CONTRIBUTIONS

S. K. and T. S. conceived and designed the study. S. K. performed the cultivation experiments, PceA characterization in crude extracts, norcobamide purification from whole cells, and subsequent HPLC analysis as well as the *Sm*CobT production, purification, and characterization. C. K. purified the PceA enzyme and analyzed its cofactor content. M. B. performed the X-ray structural analysis of PceA equipped with the different norcobamides. C. P. resolved the structure of the various norcobamides via NMR techniques. R. C. M. identified the norcobamides and the products of the *Sm*CobT assay by MS. S. K. and T. S. drafted the manuscript. All authors participated in data analysis, discussion, and writing of the manuscript.

GRAPHICAL ABSTRACT



ABBREVIATED SUMMARY

The tetrachloroethene reductive dehalogenase of the organohalide-respiring *Sulfurospirillum multivorans* harbors a norcobamide cofactor at the active site. Structural changes in the norcobamide's lower base can lead to an inactive enzyme. This study sheds light on the molecular basis underlying the structural diversity of norcobamides in the organism and visualizes the enzyme-cofactor interplay at an atomic level. Conclusions were drawn on the selectivity of cobamide-containing RDases for structurally different cobamide cofactors.

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SUPPORTING INFORMATION

Supplementary Table 1. Data collection and refinement statistics

	PceA harboring **6-OHBza**-norcobamide	PceA harboring **5-OMeBza**-norcobamide
Data Collection	PETRA III P11	HZB-MX 14.1
Wavelength	0.9184	0.9184
Resolution range	36.1 - 1.59 (1.65 - 1.59)	39.8 - 1.60 (1.66 - 1.60)
Space group	*P* 4_1	*P* 4_1
Unit cell (Å, °)	73.6 73.6 184.7 90 90 90	73.6 73.6 185.3 90 90 90
Unique reflections	129361 (12146)	129127 (12767)
Multiplicity	13.2 (12.6)	13.8 (13.6)
Completeness (%)	99 (93)	100 (99)
I/sigma(I)	14.9 (1.9)	19.0 (1.7)
Wilson B-factor	19.0	20.2
R-merge	0.114 (0.931)	0.098 (1.57)
R-pim	0.033 (0.270)	0.027 (0.436)
$CC_{1/2}$	1.00 (0.80)	1.00 (0.62)
Refinement		
R-work	0.152 (0.257)	0.151 (0.260)
R-free	0.177 (0.285)	0.172 (0.289)
Number of non-H atoms	7945	7820
macromolecules	6838	6843
ligands	243	251
solvent	864	726
Protein residues	862	865
RMS(bonds, Å)	0.007	0.007
RMS(angles, °)	0.93	0.93
Ramachandran favored (%)	98	98
Ramachandran outliers (%)	0	0
Rotamer outliers (%)	0	0
Clashscore	2.7	2.4
Average B-factor	26.5	26.2
macromolecules	25.5	25.4
ligands	21.7	21.7
solvent	36.0	35.1

NMR data (extended)

Structure analysis of the 6-OHBza-*N*-α-ribofuranosyl moiety of 6-OHBza-norcobamide. All norcobamide (NCba) structures were analyzed by NMR spectroscopy with focus on the various benzimidazoles incorporated during the cultivation experiments. In the following, the structure analysis of the 6-OHBza-*N*-α-ribofuranosyl moiety of 6-OHBza-NCba is described as a typical example (Fig. S1-8). In an analogous manner the structures and chemical shifts of the 5-OMeBza-NCba (Fig. S9-14), the 5-MeBza-NCba (Fig. S15-20), and the 6-MeBza-NCba (Fig. S21-26) have been worked out.

The ^{1}H NMR spectrum showed six signals in the low field range, three of them attributable to an aromatic AMX spin system. However, a deviation of the chemical shifts compared to the literature (Crofts *et al.* 2014) was observed and thus the structure was elucidated *de novo*. Starting point was a ^{1}H-^{13}C HSQC cross signal at δ_H 6.17, *m*/δ_C 86.6 (Fig. S6), which was assigned to position 1' of the α-ribofuranose moiety. By means of ^{1}H-^{1}H DQFCOSY, selective ^{1}H-^{1}H TOCSY (offset on H-1') and ^{1}H-^{13}C HSQC all remaining positions of the α-ribofuranose were assigned (Fig. S4) The ^{1}H-^{13}C HMBC (Fig. S7) correlation of H-1' with C-2 (δ_C 141.8) was proving the connection of the α-ribofuranosyl with the benzimidazolyl part. In order to elucidate the position of the hydroxyl group at the benzimidazole, a ^{1}H-^{1}H ROESY experiment was conducted (Fig. S8). Correlation of H-1' with the broad singlet of H-7 (δ_H 6.80) was observed, and ^{1}H-^{13}C HSQC revealed the corresponding C-7 at δ_C 96.8. Furthermore, H-4' (δ_H 4.02, *m*) showed a ROE-correlation to H-2 (δ_H 7.03), indicating that the α-ribofuranosyl and benzimidazolyl systems have a perpendicular orientation towards each other. Because no ^{1}H-^{1}H DQFCOSY correlation of H-7 was observed, the position of the hydroxyl group in the benzimidazolyl moiety had to be in position 6. The remaining protonated positions of the benzimidazole were assigned from ^{1}H-^{13}C HMBC and ^{1}H-^{1}H DQFCOSY correlations, respectively. ^{1}H-^{13}C HMBC correlations from H-7 revealed the chemical shifts of position 5 (δ_H 6.73, *d*, $^{3}J_{HH}$=8.9 Hz /δ_C 113.2) and position 9 (δ_C 132.2). H-4 (δ_H 6.49, *d*, $^{3}J_{HH}$=8.9 Hz) was determined from a ^{1}H-^{1}H DQFCOSY correlation with H-5. The corresponding ^{13}C chemical shift C-4 (δ_C 117.4) was determined by ^{1}H-^{13}C HSQC. The ^{13}C chemical shifts of position 8 (δ_C 132.5) and 6 (δ_C 153.7) were determined from the ^{1}H-^{13}C HMBC correlation with H-4. Finally, the ^{1}H-^{13}C HMBC correlation of H-2 with C-8 and C-9, the evidence for the linkage of the imidazolyl moiety with

the phenyl moiety of benzimidazole, completed the structure elucidation of the 6-OHBza-NCba.

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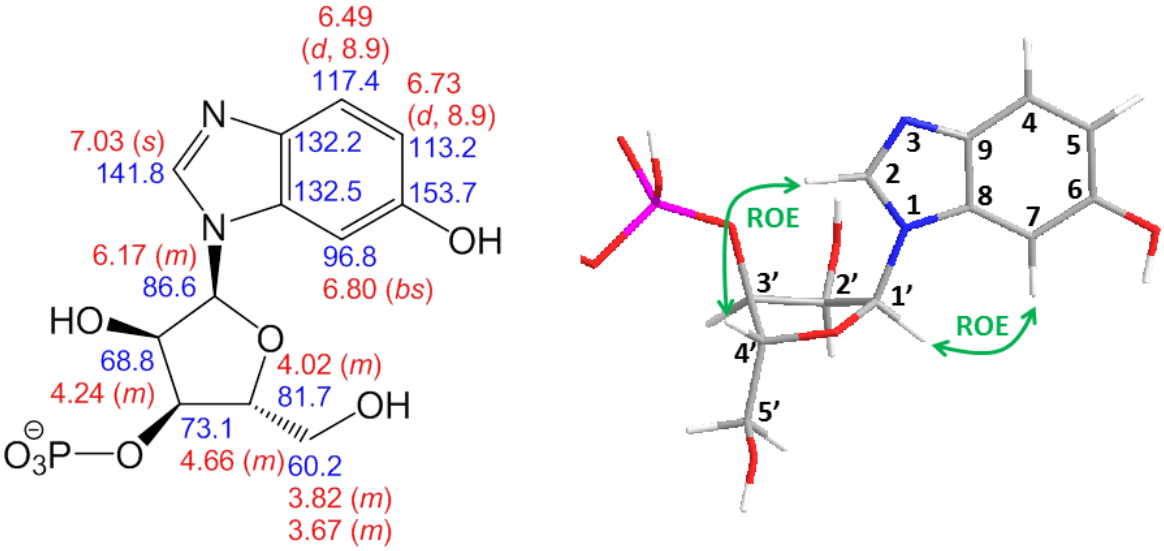

Fig. S1: Chemical shifts (δ_H red, δ_C blue) of the 6-OHBza-*N*-α-ribofuranosyl-fragment and the ^{1}H-^{1}H ROESY key correlations for determination of the position of the hydroxyl group.

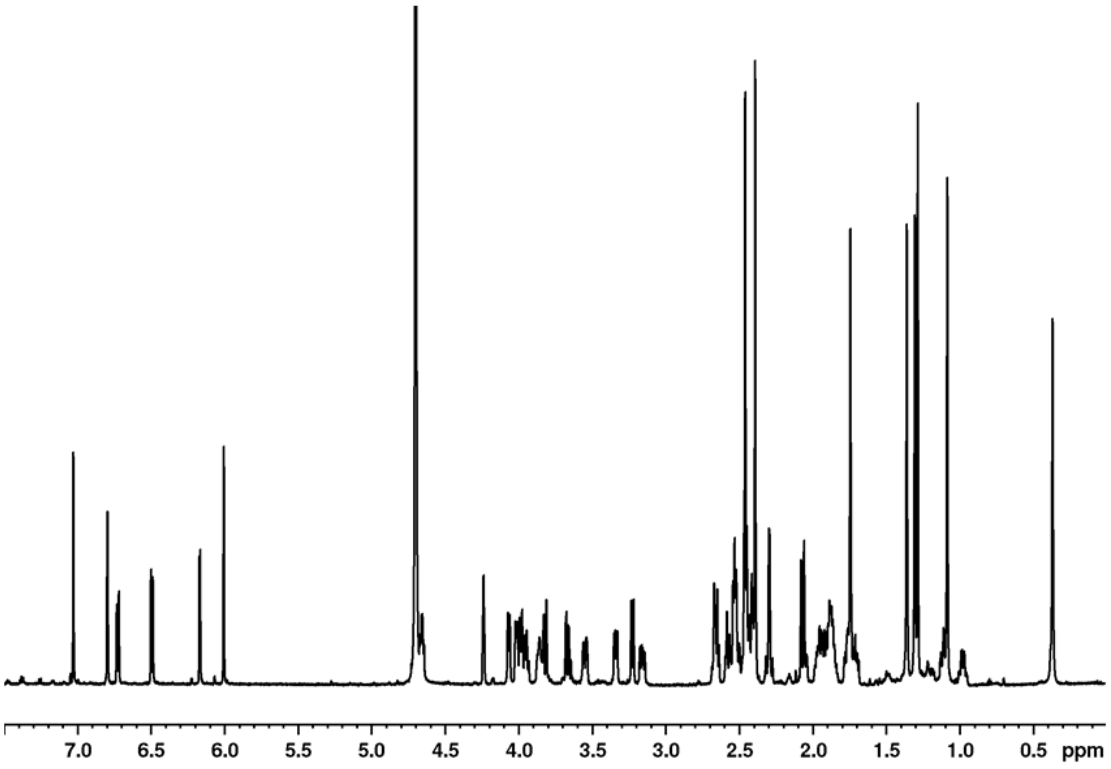

Fig. S2: ^{1}H-NMR spectrum of the 6-OHBza-NCba.

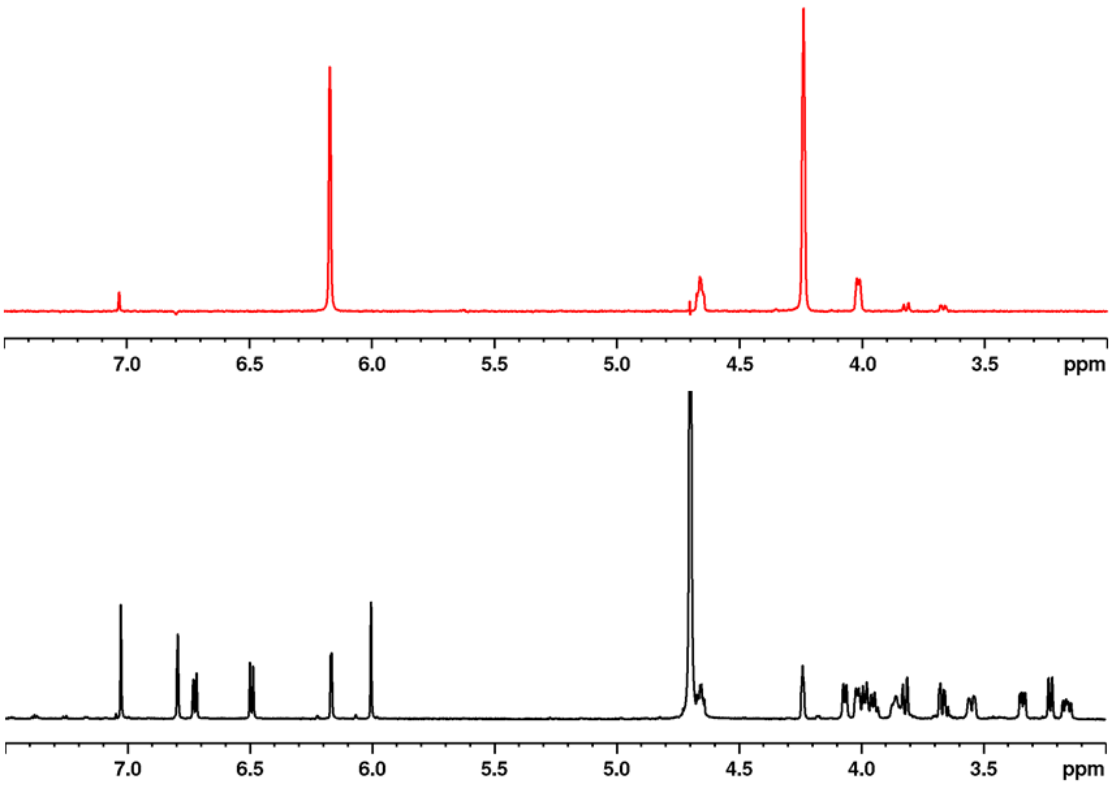

Fig. S3: ^{1}H-SELTOCSY spectrum of the α-ribofuranosyl moiety (red) of the 6-OHBza-NCba compared to the ^{1}H-NMR spectrum (black) of the 6-OHBza-NCba.

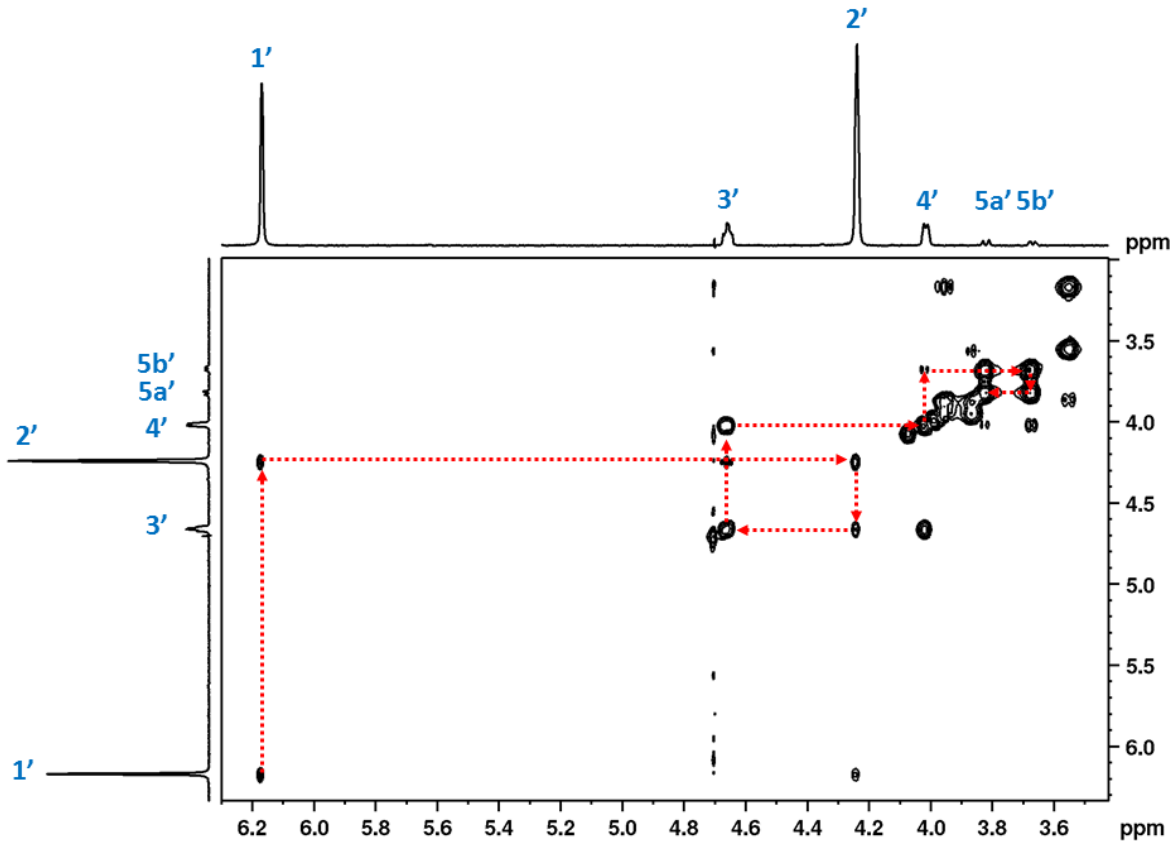

Fig. S4: ^{1}H-^{1}H DQFCOSY spectral detail of the α-ribofuranosyl unit of 6-OHBza-NCba with SELTOCSY spectra as projections.

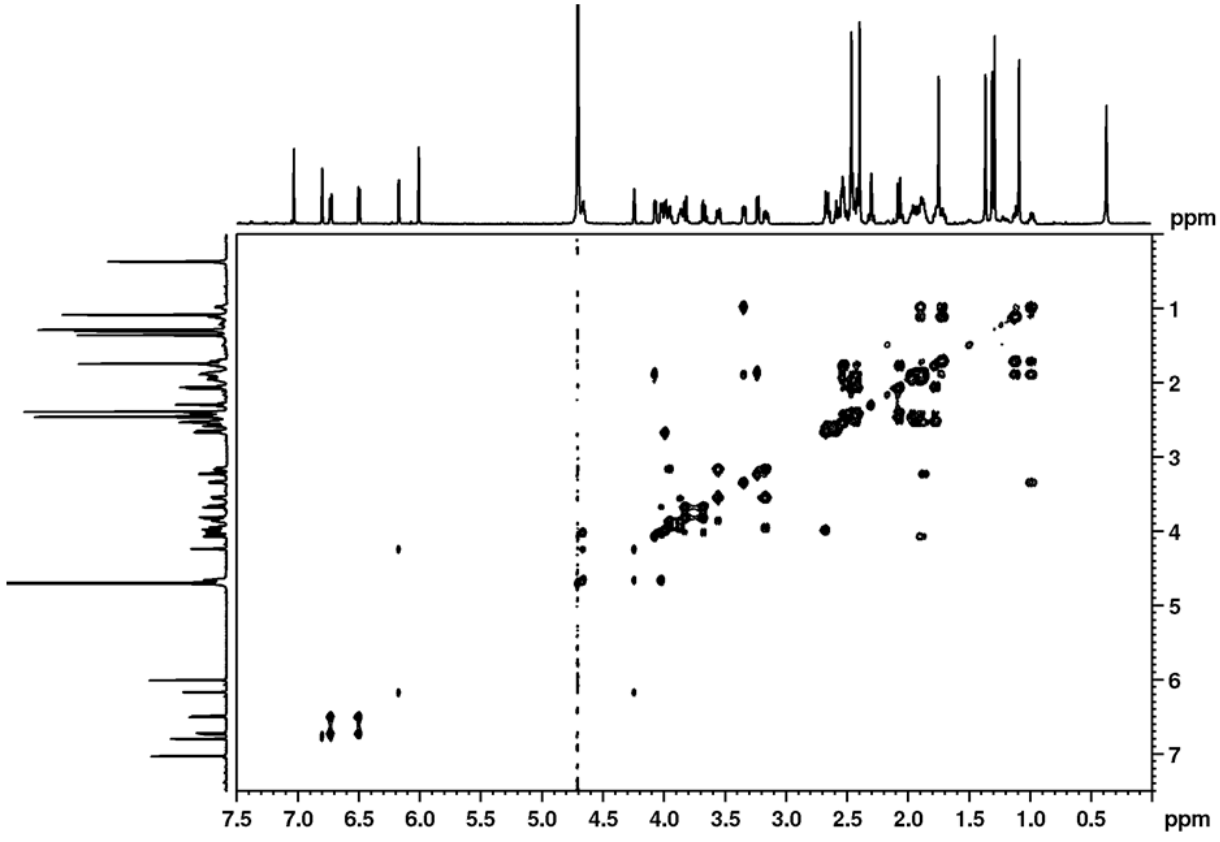

Fig. S5: Full ^{1}H-^{1}H-DQFCOSY spectrum of the 6-OHBza-NCba.

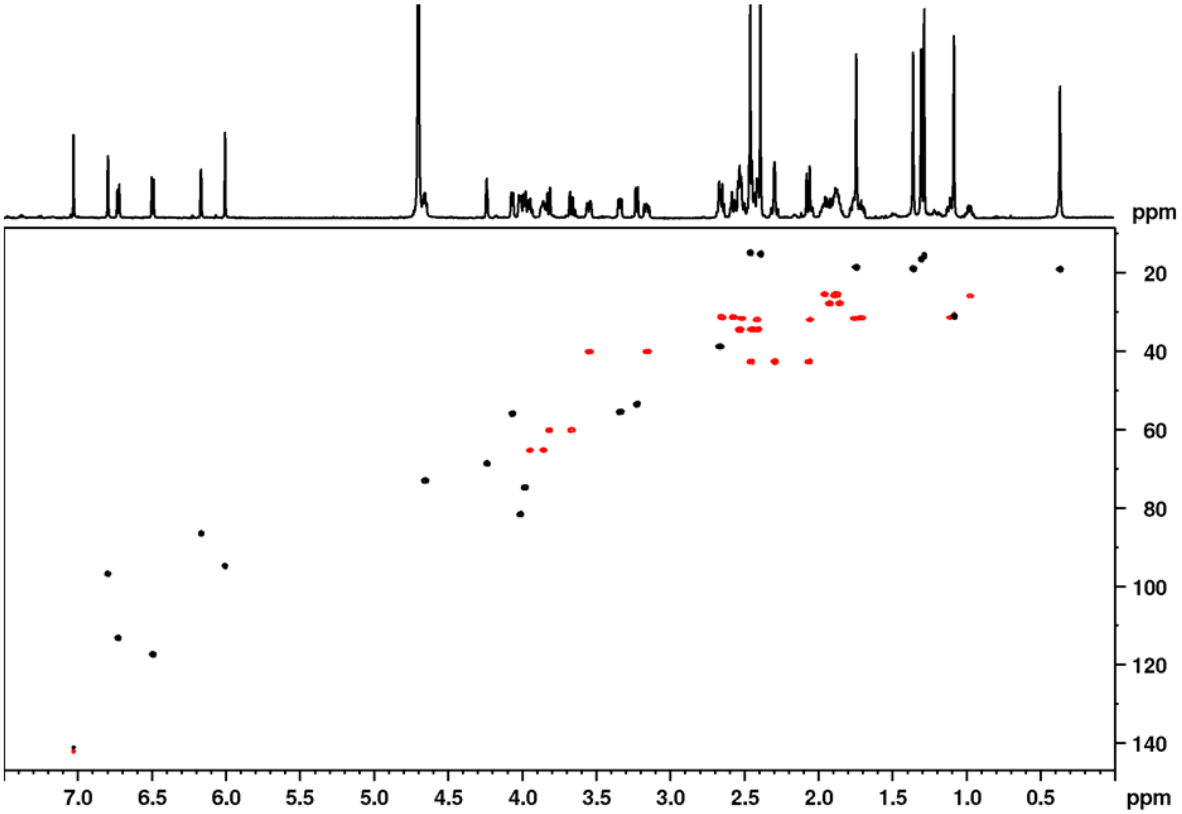

Fig. S6: ^{1}H-^{13}C HSQC spectrum of 6-OHBza-NCba.

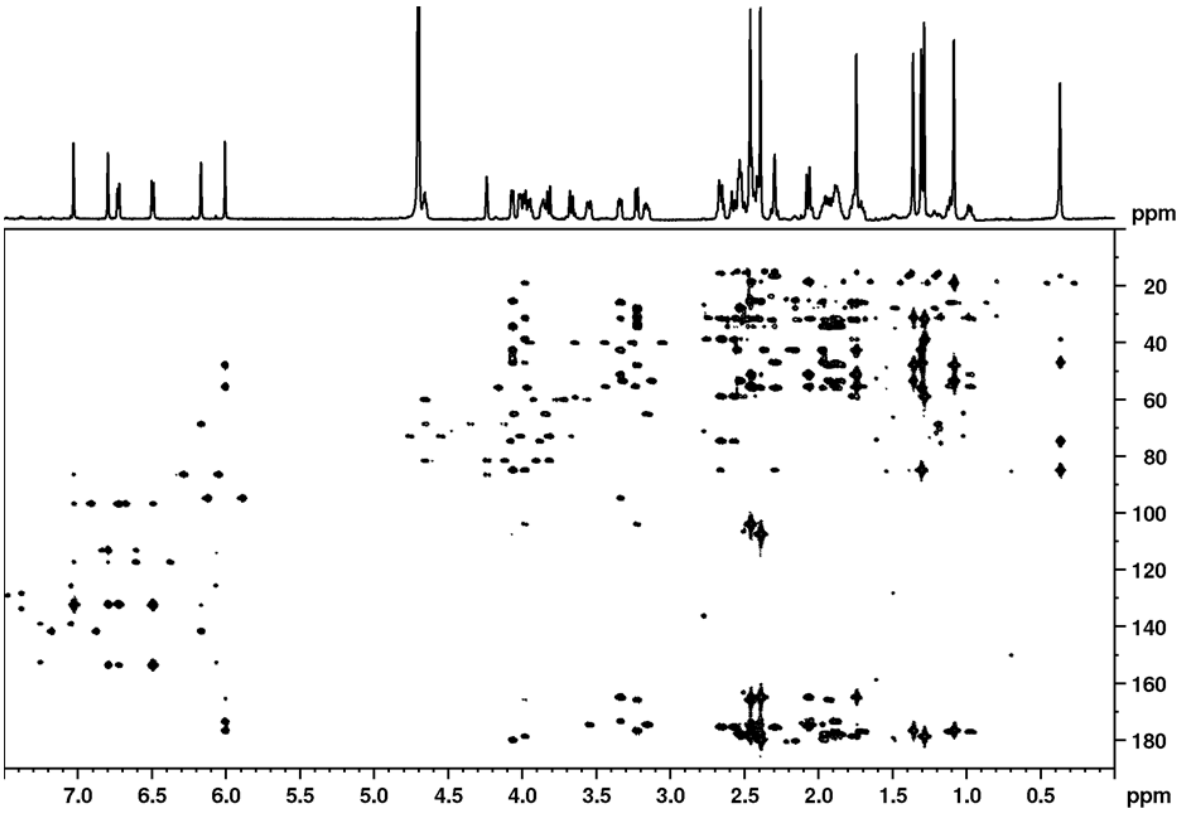

Fig. S7: ^{1}H-^{13}C HMBC spectrum of the 6-OHBza-NCba.

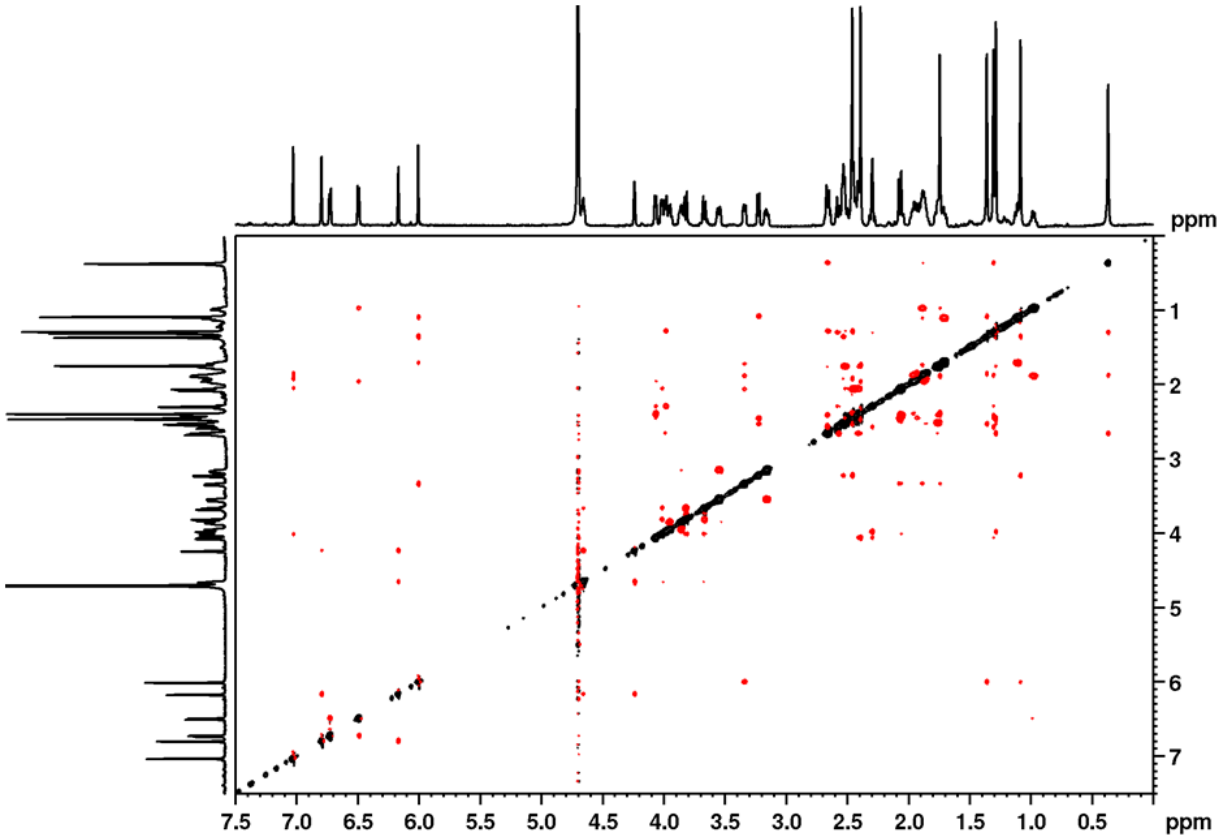

Fig. S8: ^{1}H-^{1}H ROESY spectrum of the 6-OHBza-NCba.

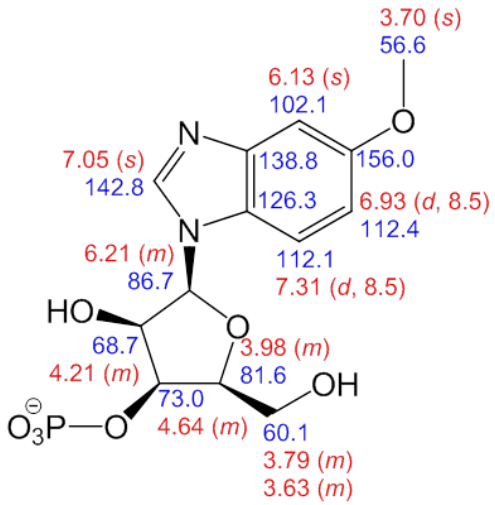

Fig. S9: Chemical shifts (δ_H red, δ_C blue) of the 5-OMeBza-*N*-α-ribofuranosyl-fragment.

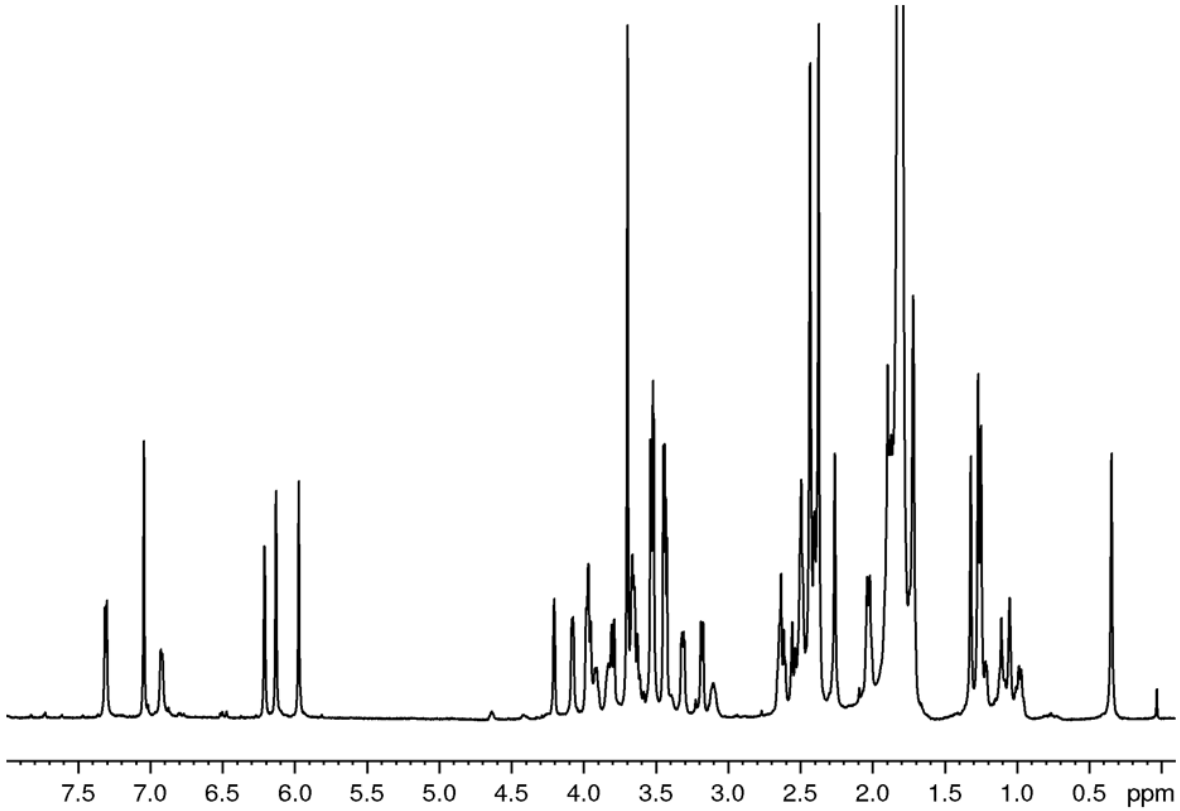

Fig. S10: ^{1}H-NMR spectrum of the 5-OMeBza-NCba.

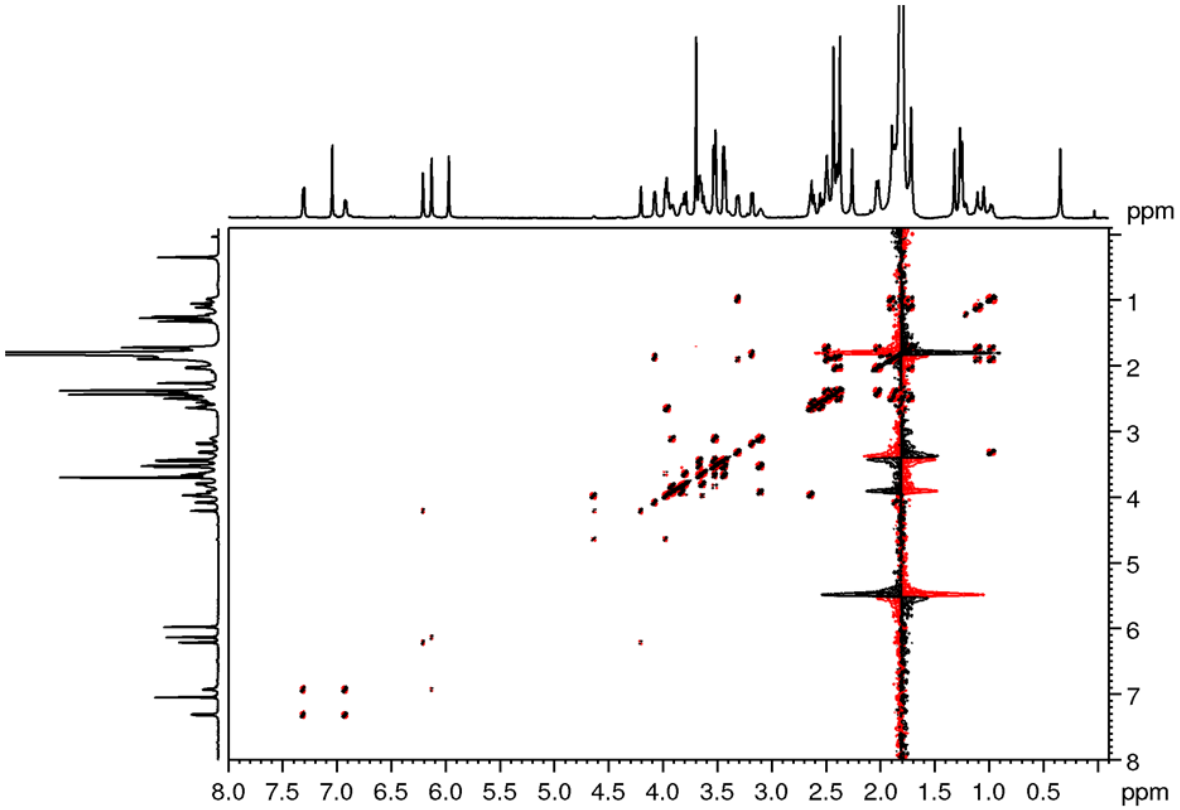

Fig. S11: ^{1}H-^{1}H-DQFCOSY spectrum of the 5-OMeBza-NCba.

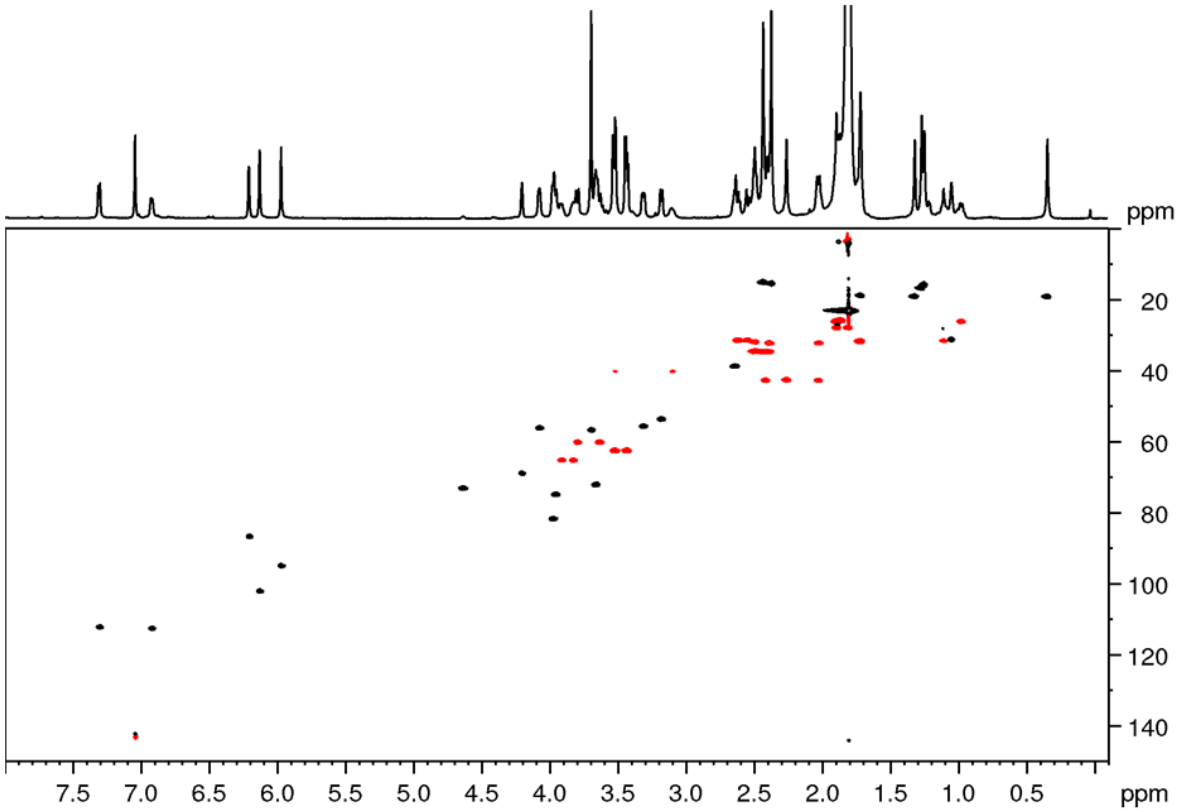

Fig. S12: ^{1}H-^{13}C HSQC spectrum of 5-OMeBza-NCba.

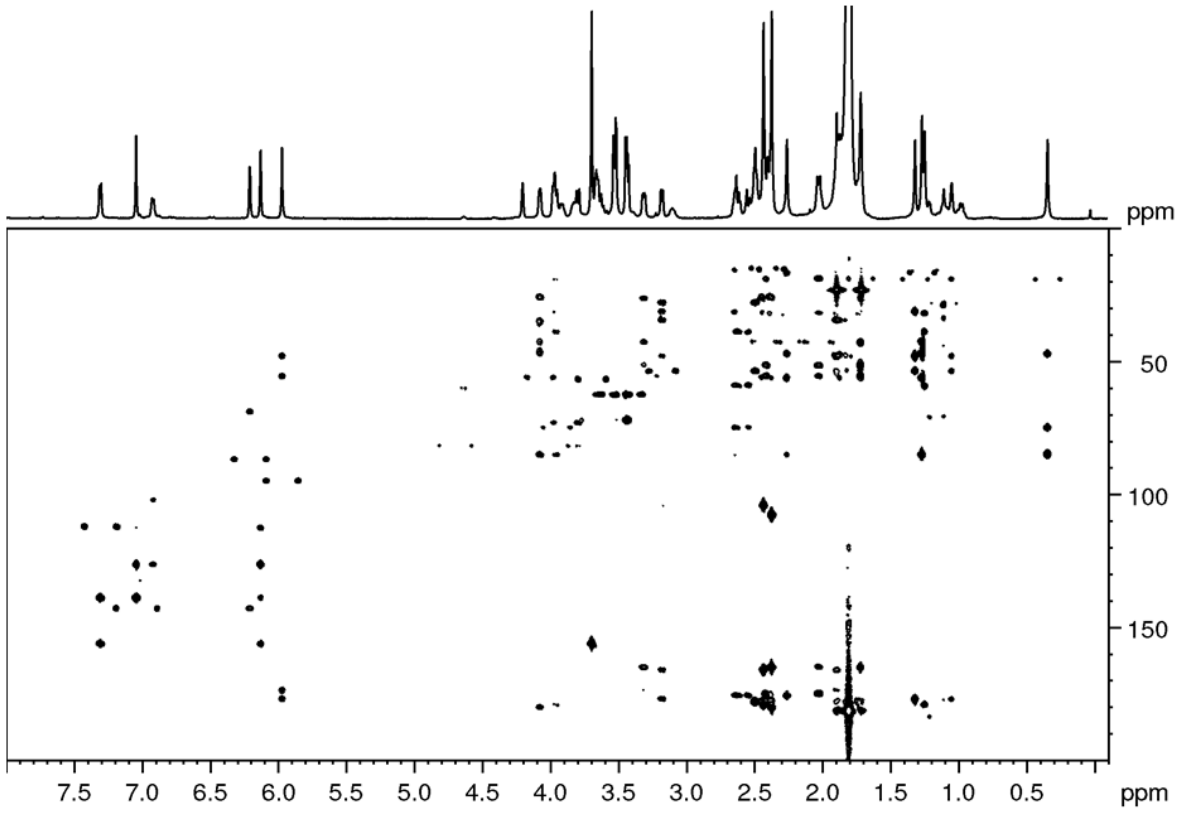

Fig. S13: ^{1}H-^{13}C HMBC spectrum of the 5-OMeBza-NCba.

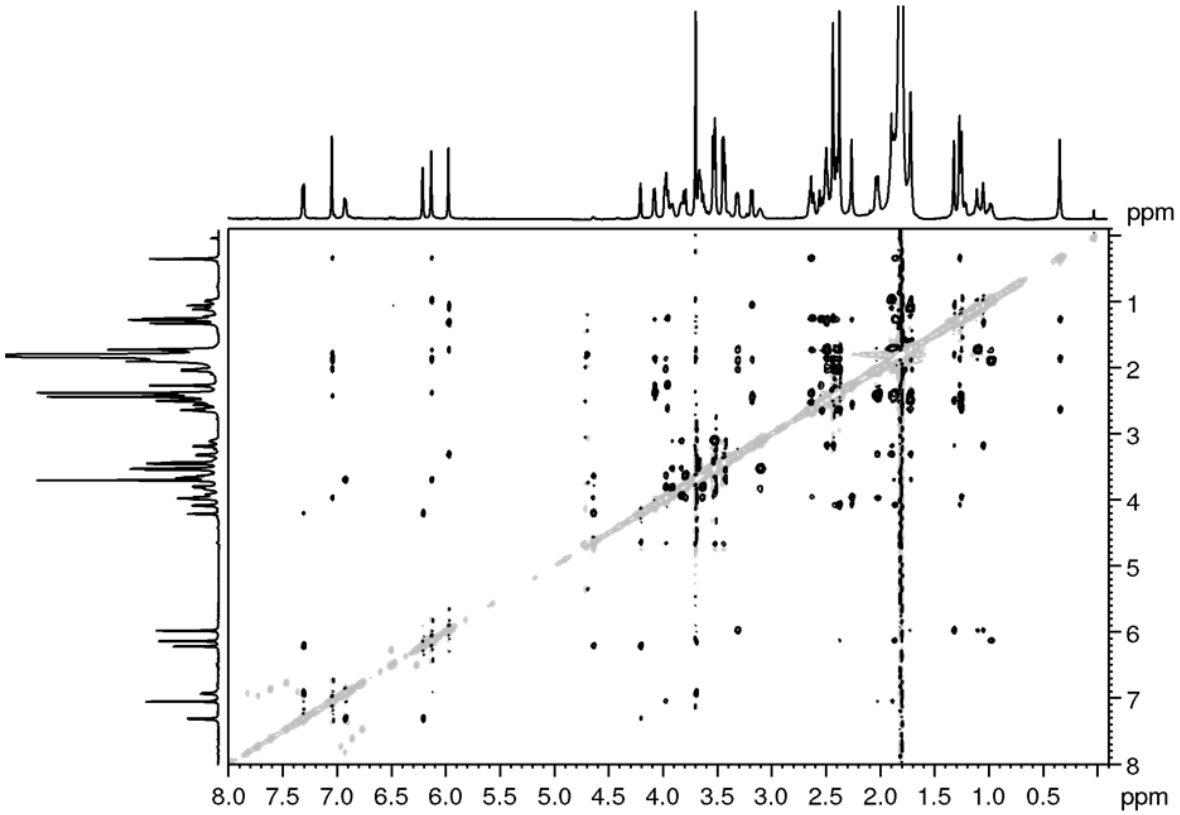

Fig. S14: ^{1}H-^{1}H ROESY spectrum of the 5-OMeBza-NCba

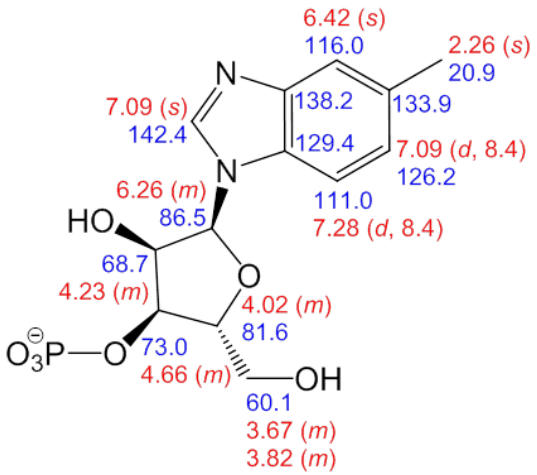

Fig. S15: Chemical shifts (δ_H red, δ_C blue) of the 5-MeBza-*N*-α-ribofuranosyl-fragment.

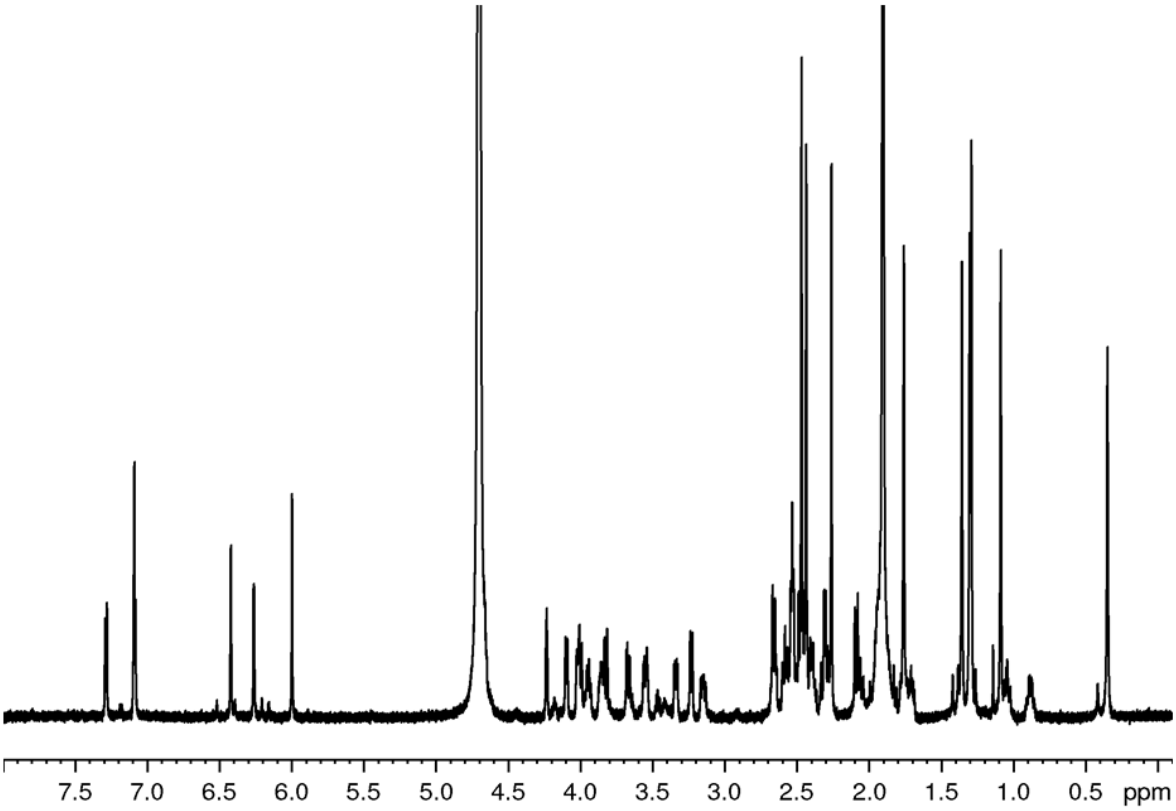

Fig. S16: ^{1}H-NMR spectrum of the 5-MeBza-NCba.

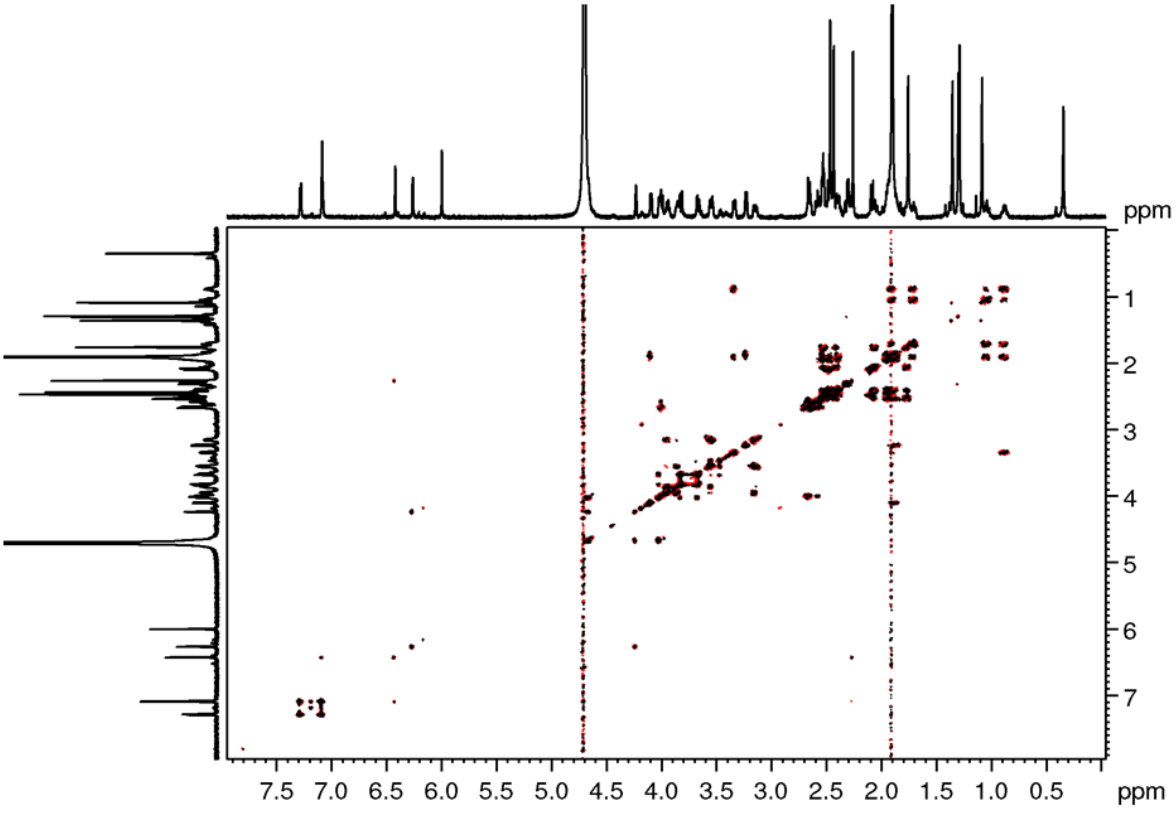

Fig. S17: ^{1}H-^{1}H-DQFCOSY spectrum of the 5-MeBza-NCba.

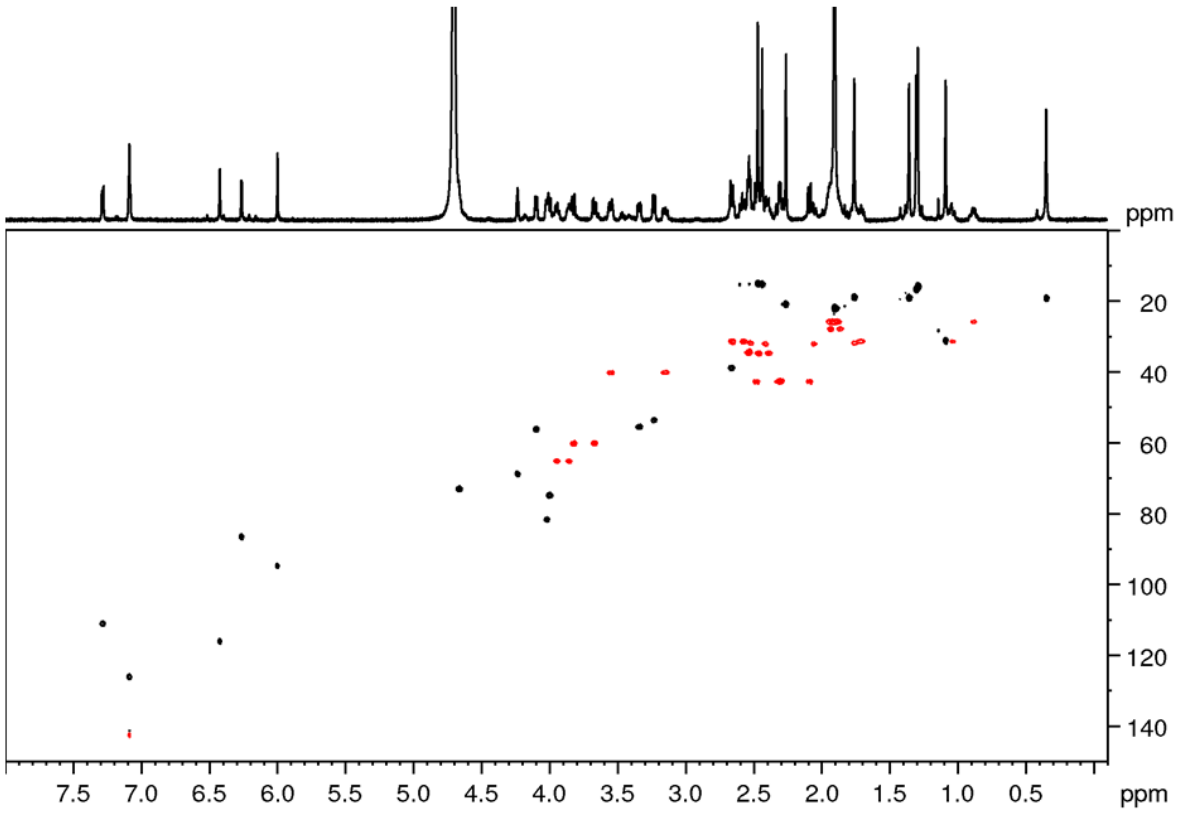

Fig. S18: ^{1}H-^{13}C HSQC spectrum of 5-MeBza-NCba.

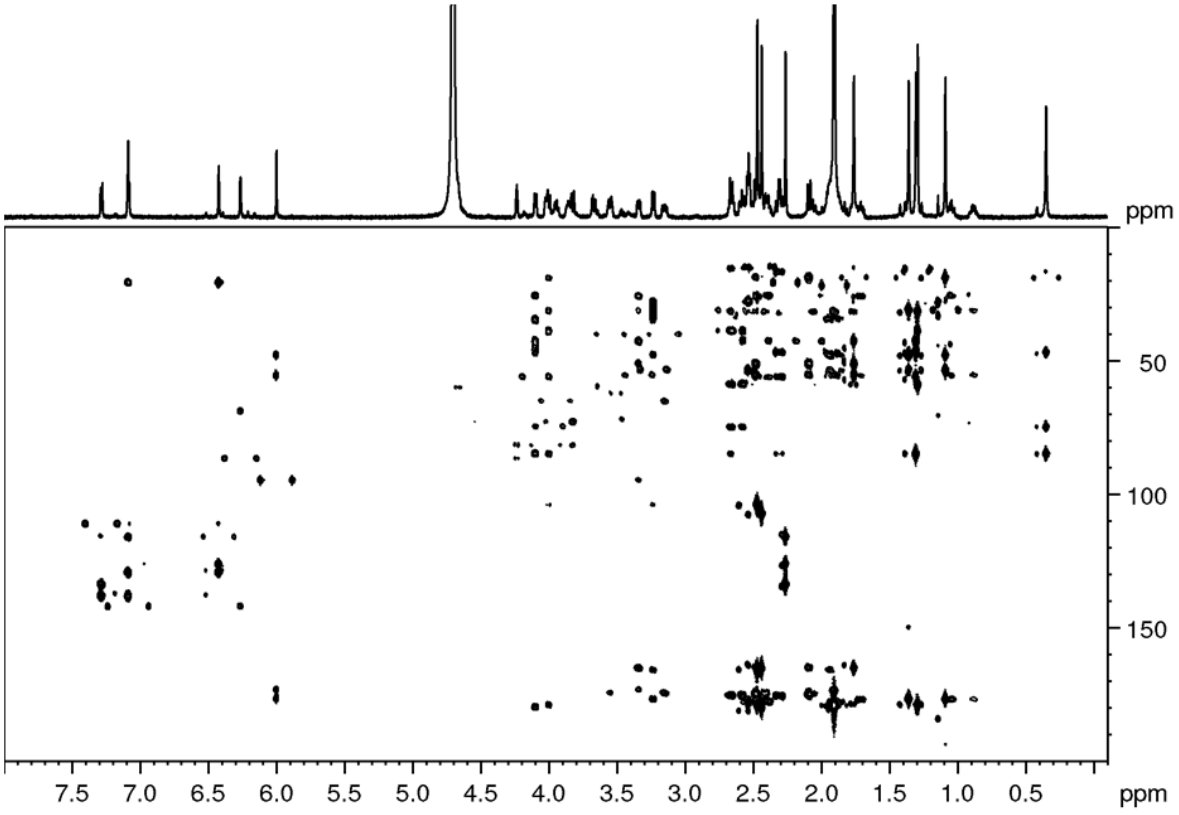

Fig. S19: ^{1}H-^{13}C HMBC spectrum of the 5-MeBza-NCba.

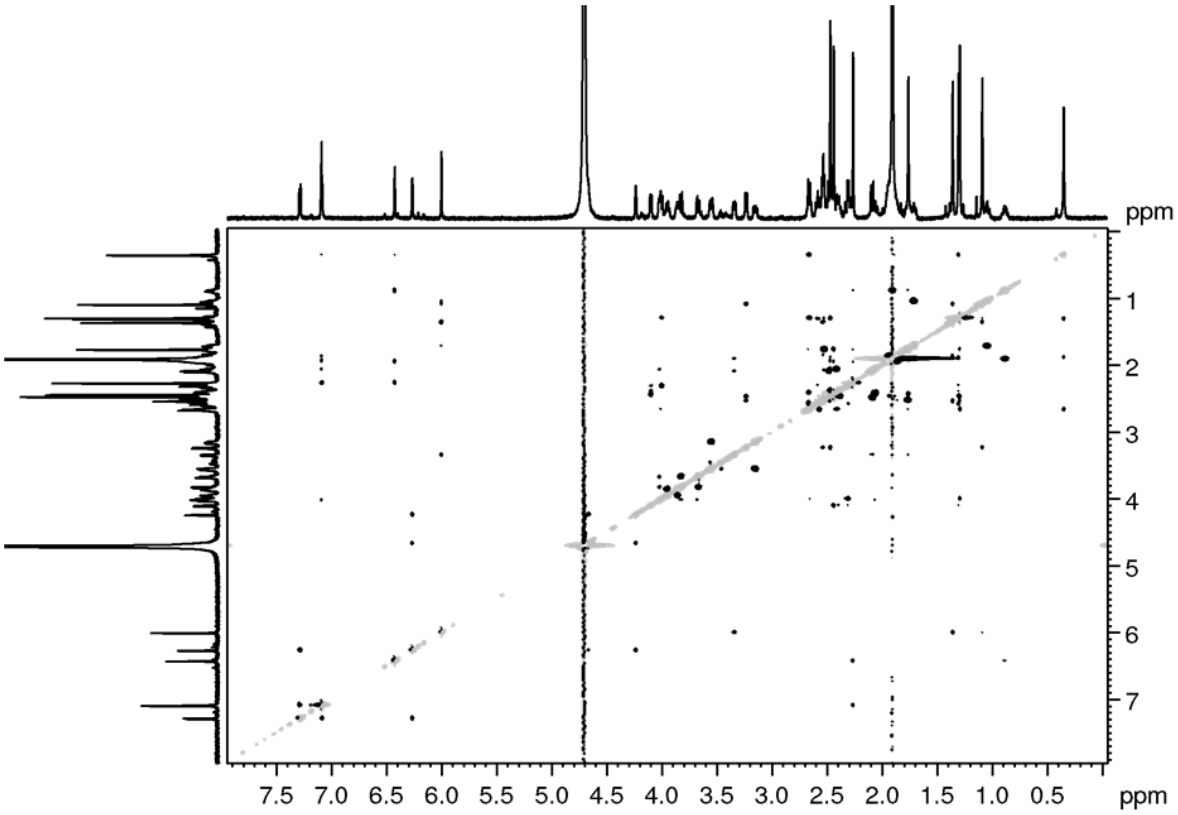

Fig. S20: ^{1}H-^{1}H ROESY spectrum of the 5-MeBza-NCba.

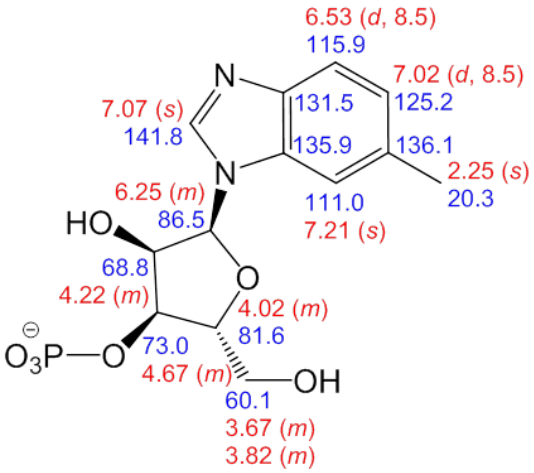

Fig. S21: Chemical shifts (δ_H red, δ_C blue) of the 6-MeBza-*N*-α-ribofuranosyl-fragment.

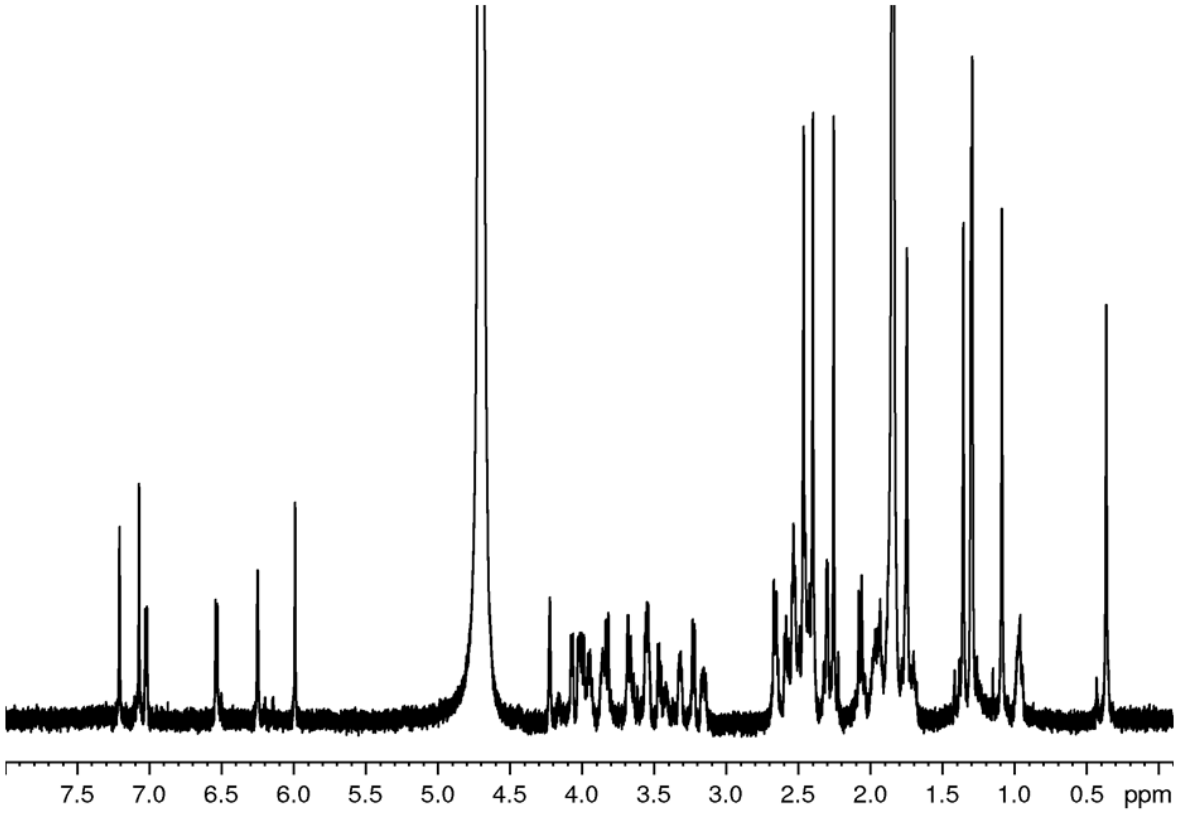

Fig. S22: ^{1}H-NMR spectrum of the 6-MeBza-NCba.

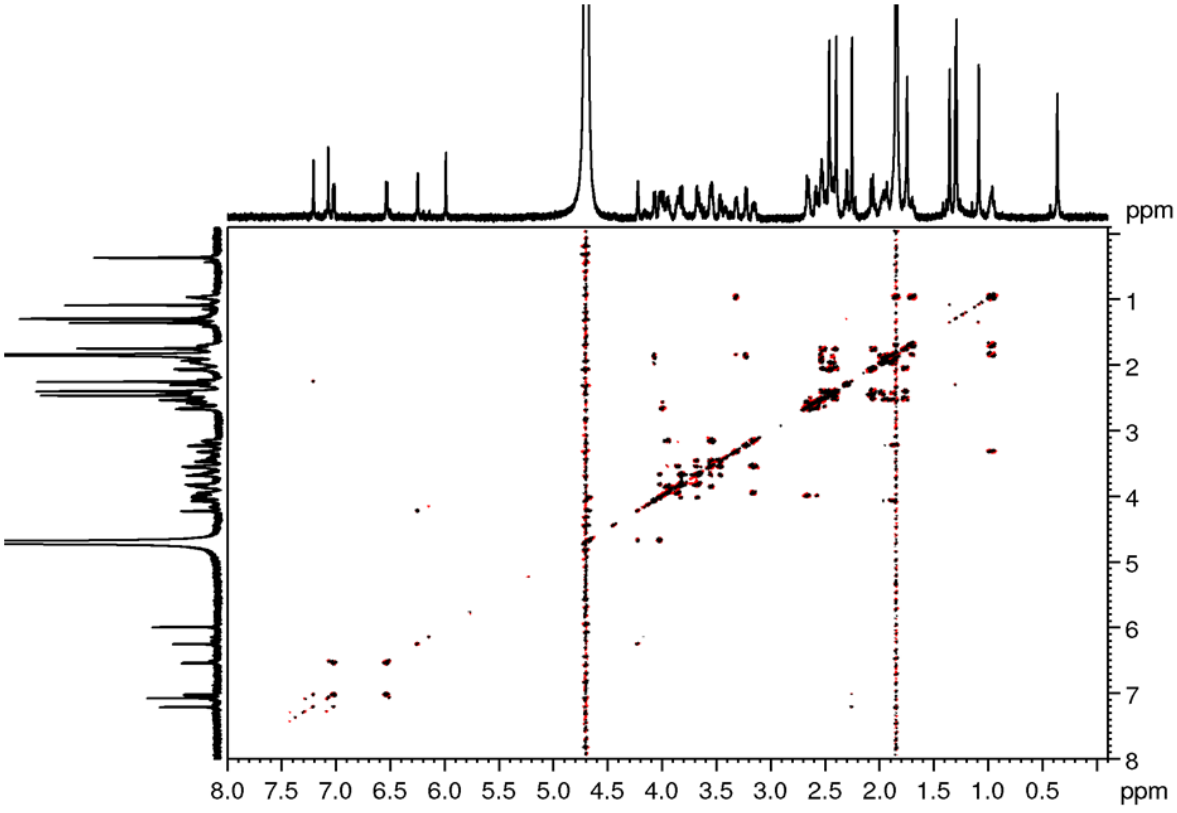

Fig. S23: ^{1}H-^{1}H-DQFCOSY spectrum of the 6-MeBza-NCba.

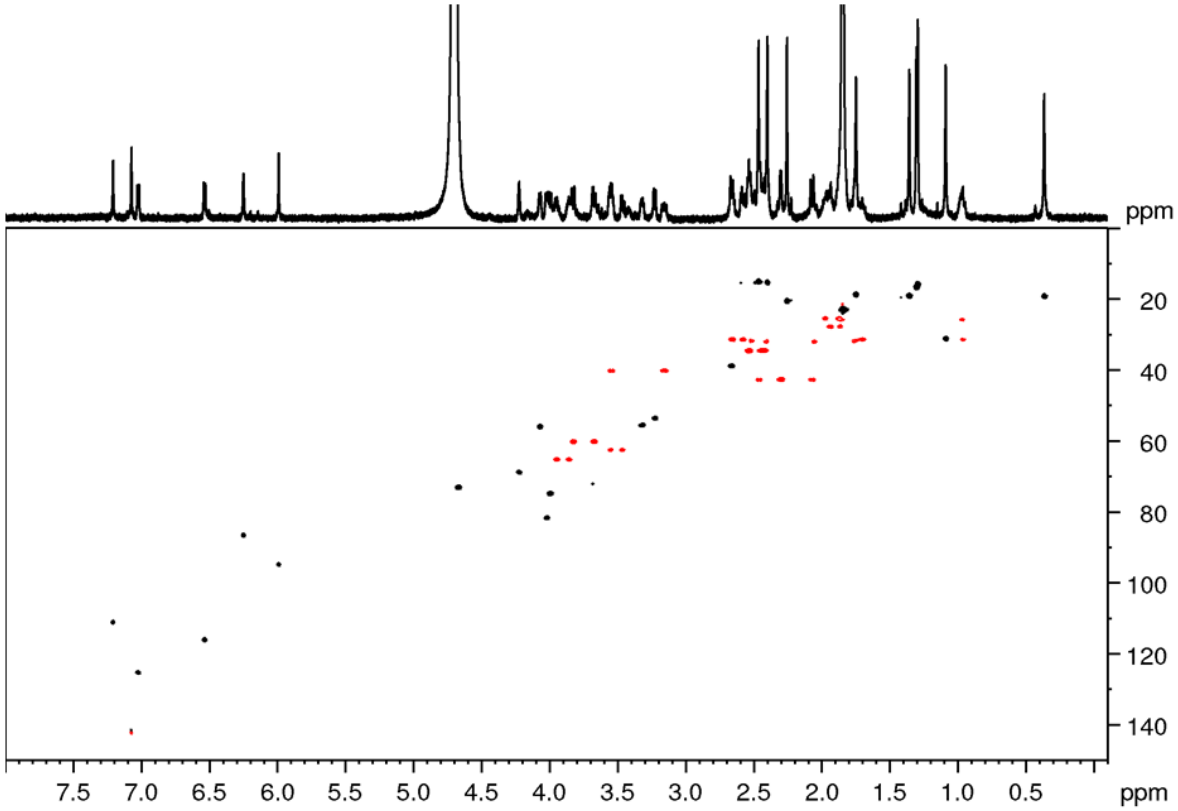

Fig. S24: ^{1}H-^{13}C HSQC spectrum of 6-MeBza-NCba.

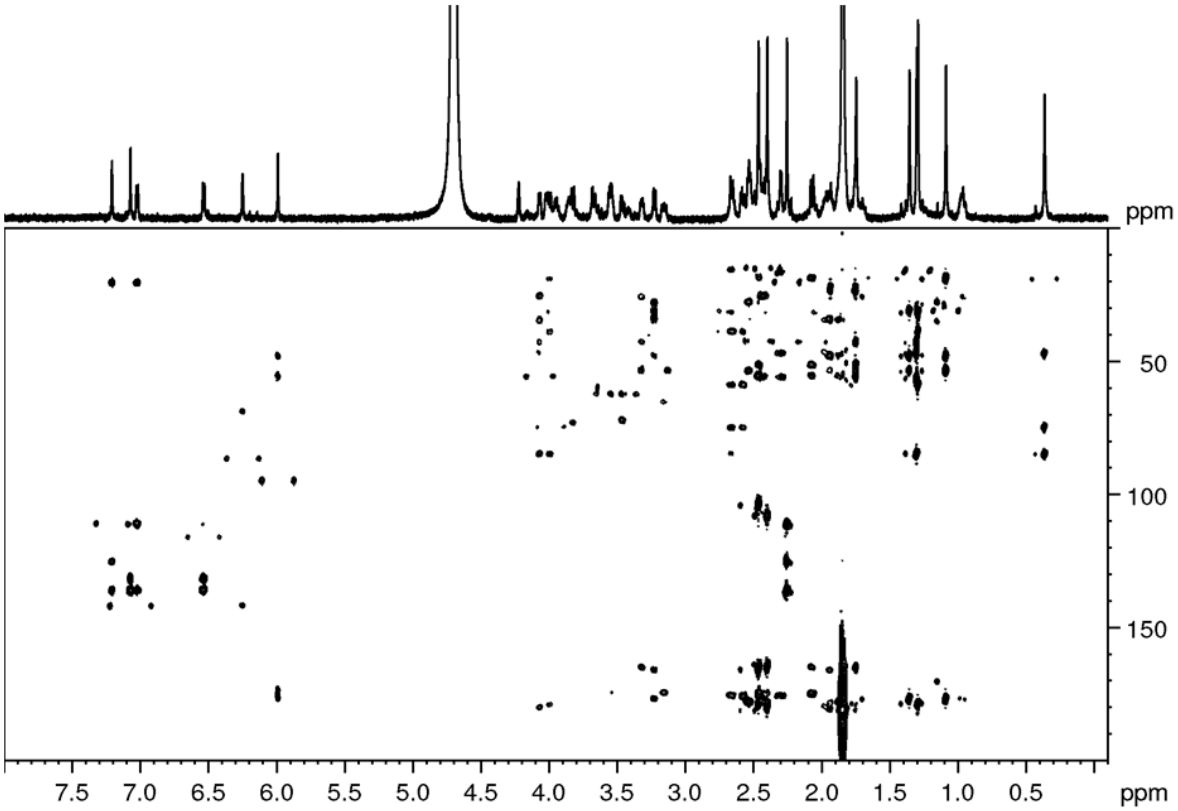

Fig. S25: ^{1}H-^{13}C HMBC spectrum of the 6-MeBza-NCba.

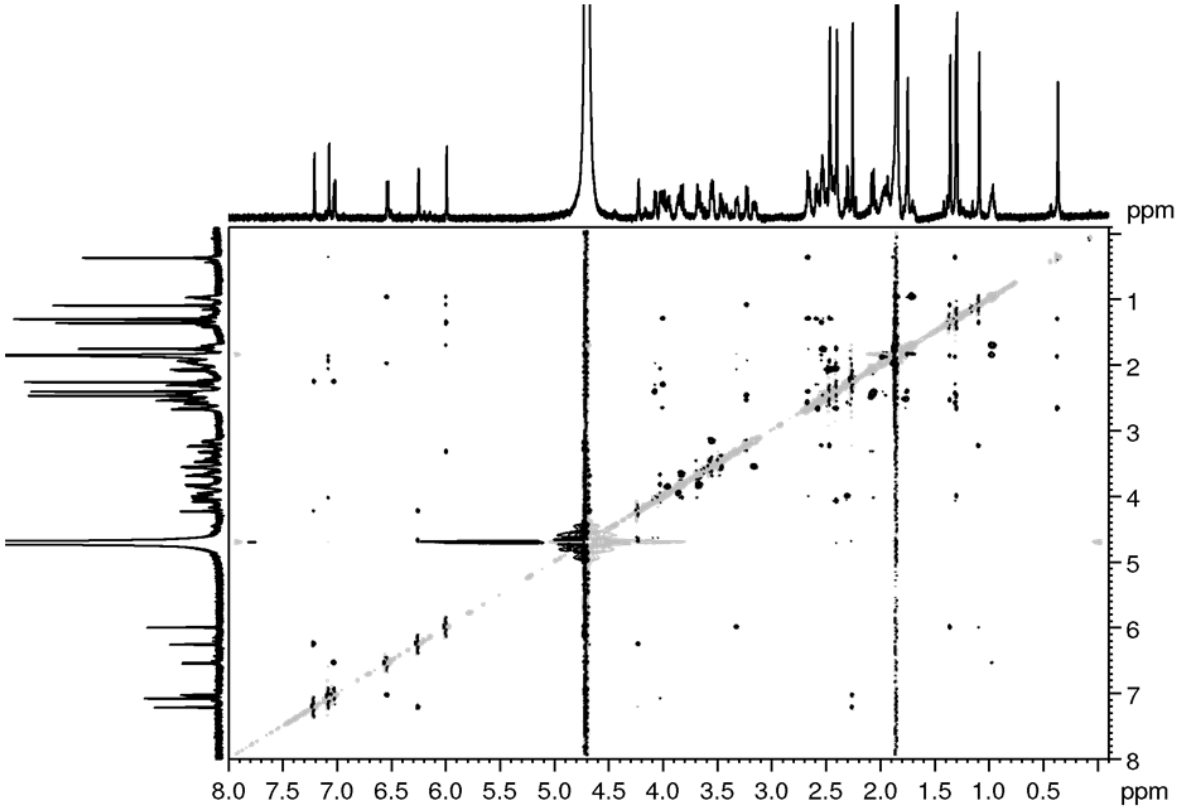

Fig. S26: ^{1}H-^{1}H ROESY spectrum of the 6-MeBza-NCba.

2.3 The *SMUL_1544* Gene Product Governs Norcobamide Biosynthesis in the Tetrachloroethene-Respiring Bacterium *Sulfurospirillum multivorans*

Sebastian Keller, Aaron Treder, Stephan H. von Reuss, Jorge C. Escalante-Semerena, Torsten Schubert

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Status of the manuscript: published

The heterologous expression of SMUL_1544 in a *Salmonella enterica* Δ*cobD* strain resulted in the predominant synthesis of norpseudo-B_{12} revealing SMUL_1544 as the ethanolamine *O*-phosphate (EA-P) linker forming enzyme in *S. multivorans*. Pseudo-B_{12} with an (*R*)-1-aminopropan-2-ol *O*-2-phosphate (AP-P) linker was the major cobamide in *S. multivorans*, when grown in the presence of 1 mM exogenous L-threonine *O*-3-phosphate (L-Thr-P), which had negative effects on the activity and the overall amount of PceA resulting in a delayed growth on PCE-medium. With help of an isotopic labelling experiment with exogenous L-[3-^{13}C]-serine, the amino acid was detected as possible, but unlike origin of EA-P in the norcobamide linker biosynthesis in *S. multivorans*.

Contribution of Sebastian Keller to this study: 80 %

Sebastian Keller performed all genetics and the growth experiments, cobamide extractions, and HPLC analysis from *S. enterica* (Fig. 2, 3). He performed the cobamide extraction, growth studies, investigation of the PceA activity and maturation in *S. multivorans amended* with L-Thr-P (Fig. 4, 5). He cultivated and isolated the cobamide fraction with L-[3-^{13}C]-serine as preparation for the NMR analysis and he contributed to the writing of this manuscript.

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The *SMUL_1544* Gene Product Governs Norcobamide Biosynthesis in the Tetrachloroethene-Respiring Bacterium *Sulfurospirillum multivorans*

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ABSTRACT

The tetrachloroethene (PCE)-respiring bacterium *Sulfurospirillum multivorans* produces a unique cobamide, namely, norpseudo-B_{12}, which, in comparison to other cobamides, e.g., cobalamin and pseudo-B_{12}, lacks the methyl group in the linker moiety of the nucleotide loop. In this study, the protein SMUL_1544 was shown to be responsible for the formation of the unusual linker moiety, which is most probably derived from ethanolamine-phosphate (EA-P) as the precursor. The product of the *SMUL_1544* gene successfully complemented a *Salmonella enterica* Δ*cobD* mutant. The *cobD* gene encodes an L-threonine-*O*-3-phosphate (L-Thr-P) decarboxylase responsible for the synthesis of (*R*)-1-aminopropan-2-ol *O*-2-phosphate (AP-P), required specifically for cobamide biosynthesis. When SMUL_1544 was produced in the heterologous host lacking CobD, norpseudo-B_{12} was formed, which pointed toward the formation of EA-P rather than AP-P. Guided cobamide biosynthesis experiments with minimal medium supplemented with L-Thr-P supported cobamide biosynthesis in *S. enterica* producing SMUL_1544 or *S. multivorans*. Under these conditions, both microorganisms synthesized pseudo-B_{12}. This observation indicated a flexibility in the SMUL_1544 substrate spectrum. From the formation of catalytically active PCE reductive dehalogenase (PceA) in *S. multivorans* cells producing pseudo-B_{12}, a compatibility of the respiratory enzyme with the cofactor was deduced. This result might indicate a structural flexibility of PceA in cobamide binding. Feeding of L-[3-^{13}C]serine to cultures of *S. multivorans* resulted in isotope labeling of the norpseudo-B_{12} linker moiety, which strongly supports the hypothesis of EA-P formation from L-serine-*O*-phosphate (L-Ser-P) in this organism.

IMPORTANCE

The identification of the gene product SMUL_1544 as a putative L-Ser-P decarboxylase involved in norcobamide biosynthesis in *S. multivorans* adds a novel module to the assembly line of cobamides (complete corrinoids) in prokaryotes. Selected cobamide-containing enzymes (e.g., reductive dehalogenases) showed specificity for their cobamide cofactors. It has recently been proposed that the structure of the linker moiety of norpseudo-B_{12} and the mode of binding of the EA-P linker to the PceA enzyme reflect the high specificity of the enzyme for its cofactor. Data reported herein do not support this idea. In fact, norpseudo-B_{12} was functional in the cobamide-dependent methionine biosynthesis of *S. enterica*, raising questions about the role of norcobamides in nature.

Cobamides, such as cobalamin (also known as Cbl or, in its cyano form, as vitamin B_{12}), are complex natural products exclusively produced by prokaryotes (1). They act as essential cofactors of a defined set of enzymes that are either mutases, eliminases, methyltransferases, or reductive dehalogenases (2, 3). Cobamides share a complex core structure, the corrin ring, which is a unique contracted tetrapyrrole ring system that binds a central cobalt ion (4). When the cobalt ion is in its Co^{3+} oxidation state, it binds two additional ligands. In natural cobamides, different upper ligands, e.g., a 5′-deoxyadenosyl moiety, a methyl group, or a hydroxyl group, have been identified on the β-face of the corrin ring. Purines (e.g., adenine) or benzimidazoles serve as the lower ligand (on the α-face of the corrin ring) (5). Structural diversity among cobamides is limited to variations in the type of upper and lower ligands and, in one special case, a modification to the linker moiety of the nucleotide loop (6). The lower ligand is part of the nucleotide loop tethering the ligand base to the corrin ring. In almost all cobamides, a linker unit derived from (*R*)-1-aminopropan-2-ol *O*-2-phosphate (AP-P) connects the nucleotide to the D ring of the cobalt porphinoid. The assembly of the nucleotide loop is the final part of the multistep cobamide biosynthesis (1). In *Salmonella enterica* serovar Typhimurium strain LT2, two enzymes are responsible for the synthesis of AP-P from L-threonine (L-Thr). The *S. enterica pduX* gene encodes the L-Thr kinase (PduX, EC 2.7.1.177), involved in cobamide biosynthesis (7); PduX generates L-threonine-*O*-3-phosphate (L-Thr-P) from L-Thr at the expense of ATP. In the second step, L-Thr-P is decarboxylated by the L-threonine-*O*-3-phosphate decarboxylase (CobD, EC 4.1.1.81), yielding AP-P (Fig. 1) (8). Finally, the

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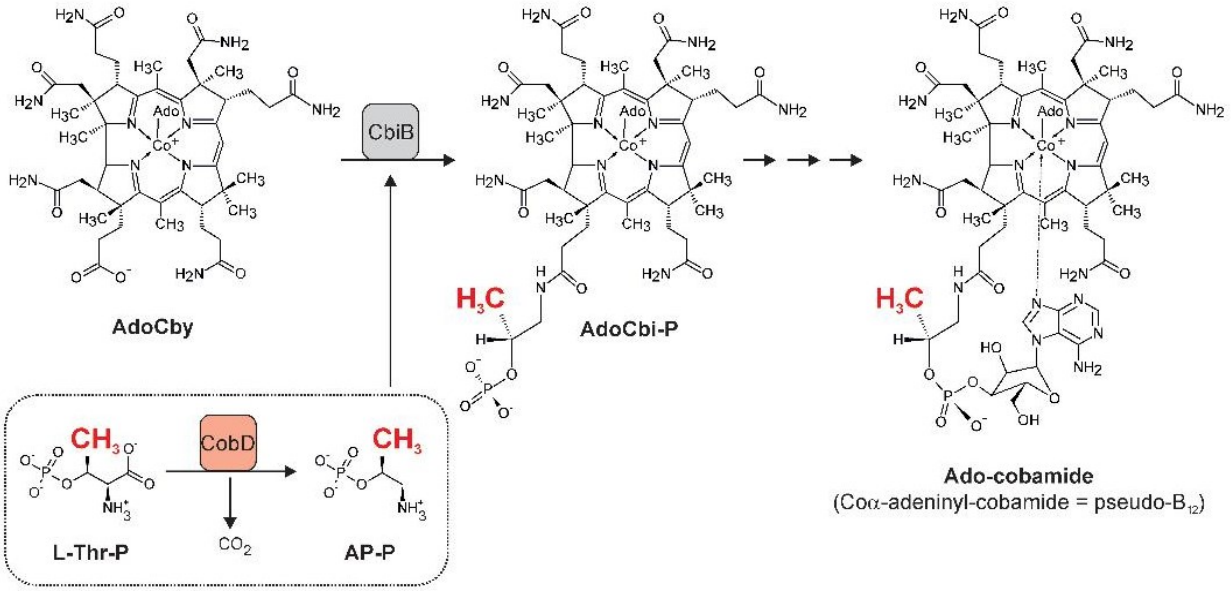

FIG 1 Schematic representation of the generation and incorporation of the cobamide linker moiety in *S. enterica*. The methyl group at position 176 that is absent in norcobamides but present in the cobamide structure is highlighted in red. L-Thr-P, L-threonine-*O*-3-phosphate; AP-P, (*R*)-1-aminopropan-2-ol *O*-2-phosphate; AdoCby, adenosylcobyric acid; AdoCbi-P, adenosylcobinamide-phosphate.

adenosylcobinamide-phosphate (AdoCbi-P) synthase (CbiB, EC 6.3.1.10) combines AP-P and adenosylcobyric acid (AdoCby) to yield adenosylcobinamide-phosphate (AdoCbi-P) (9).

CobD homologues are ubiquitously present in the genomes of cobamide-producing prokaryotes (10). The CobD decarboxylase is a pyridoxal-5′-phosphate (PLP)-dependent enzyme and shows structural similarities to PLP-dependent aminotransferases (11). The *cobD* gene product was shown to be required for the synthesis of cobamides, such as pseudo-B_{12} (Coα-adeninyl-cobamide; Fig. 1), in *S. enterica*. The growth defect caused by the absence of CobD was corrected by the addition of 1-aminopropan-2-ol (AP) to the culture medium (8, 12). AP appeared to be phosphorylated by the cells and used for cobamide biosynthesis.

The occurrence of an alternative linker unit in cobamides has been reported only for norpseudo-B_{12} (Coα-adeninyl-176-norcobamide), produced by the Gram-negative epsilonproteobacterium *Sulfurospirillum multivorans* (6). In comparison to pseudo-B_{12} (Fig. 1), norpseudo-B_{12} lacks a methyl group at position 176 in the linker moiety. It was assumed that in this special case ethanolamine-*O*-phosphate (EA-P) rather than AP-P was used as the precursor. The unusual norpseudo-B_{12} is utilized as a cofactor in the tetrachloroethene (PCE) reductive dehalogenase (PceA) in *S. multivorans* (13). The PceA enzyme functions as the terminal reductase in the organohalide respiratory metabolism of this anaerobe (14). Recently, the three-dimensional crystal structure of the PceA enzyme was solved and the mode of norpseudo-B_{12} binding was reported (15). In the structure, the norcobamide cofactor is bound deeply inside PceA in the base-off conformation, which means that the adeninyl moiety does not form a coordination bond with the cobalt ion in the center of the corrin ring. Notably, the lower ligand base is not tightly enclosed by the protein environment, and in fact, it is accessible to solvent in a cavity of the PceA structure. In contrast, the adjoining linker moiety is completely packed by the protein. The backbone of a histidine residue (His357) in the PceA structure is located near the carbon atom at position 176 in the linker moiety (15). Based on this observation, we surmised that the PceA structure might favor the binding of norcobamides rather than AP-P-containing cobamides. The methyl group at position 176 in the AP-P linker moiety of cobamides may hinder binding to the site.

The availability of the *S. multivorans* genome sequence (16) provided new impetus to efforts aimed at understanding why this bacterium makes norpseudo-B_{12} rather than pseudo-B_{12}. A single large gene cluster was identified in the organohalide respiration gene region, which encodes a complete set of cobamide biosynthesis proteins. The production of the respective gene products appeared to be triggered by the availability of PCE, which highlights the close interconnection between cobamide production and PCE respiration in this organism (17). The second gene (locus tag, *SMUL_1544*) of the cobamide biosynthesis gene cluster in *S. multivorans* showed similarity to the *cobD* gene of *Salmonella enterica* (22% sequence identity). In the study presented here, the role of the SMUL_1544 protein in norpseudo-B_{12} biosynthesis in *S. multivorans* was examined. Its putative function as a decarboxylase specifically required for norcobamide formation was investigated using an *S. enterica* Δ*cobD* mutant strain. To prove that the SMUL_1544 protein is specific for the synthesis of norcobamide, the *S. enterica* Δ*cobD* strain harboring a plasmid carrying the *SMUL_1544* gene was cultivated in the presence of L-Thr-P. The cobamide synthesized by the tester strain was identified as pseudo-B_{12}, providing valuable insights into the substrate specificity of the SMUL_1544 protein *in vivo*. Finally, the role of L-serine (L-Ser) as a putative precursor of the norpseudo-B_{12} linker moiety was investigated by isotopic labeling experiments.

TABLE 1 Bacterial strains used in this study[a]

Strain	Genotype	Source or reference
JE7088	Δ*metE2702 ara-9*	J. C. Escalante-Semerena strain collection
Derivatives of JE7088		
JE11685	pBAD24[b]	J. C. Escalante-Semerena strain collection
JE12941	Δ*cobD1371*	J. C. Escalante-Semerena strain collection
JE14935	Δ*cobD1371*/pBAD24	J. C. Escalante-Semerena strain collection
JE20971	Δ*cobD1371*/pBAD24+*SMUL_1544*	This study

[a] All strains used in this study were derivatives of *Salmonella enterica* serovar Typhimurium strain LT2.
[b] From reference 21.

MATERIALS AND METHODS

Cultivation of bacteria. *S. multivorans* (DSMZ 12446, formerly *Dehalospirillum multivorans*) was grown anaerobically in a defined mineral medium (18) in the absence of yeast extract and vitamin B_{12}. Pyruvate (40 mM) served as the electron donor, and, unless otherwise stated, PCE (nominal concentration, 10 mM) served as the terminal electron acceptor. PCE was added from a sterile stock solution (0.5 M) in hexadecane. Where indicated, L-Thr-P, L-Ser-P, L-Thr, L-Ser, or L-[3-^{13}C]serine (all chemicals were purchased from Sigma-Aldrich Chemie GmbH, Munich, Germany) was added to the medium from sterile stock solutions to a final concentration of 1 mM. Growth was monitored photometrically by measuring the optical density (OD) at 578 nm. The maximal growth rate (μ_{max}) was calculated on the basis of a logarithmic plot of the average results. Derivatives of *S. enterica* serovar Typhimurium strain LT2 were cultivated on no-carbon essential (NCE) minimal medium (19) containing glycerol (22 mM for growth studies or 55 mM for cobamide analysis) as the growth substrate under aerobic conditions. Trace minerals (20), $MgSO_4$ (1 mM), and ampicillin (100 μg/ml) were added to the medium. For growth studies, 250 μM arabinose was added, and for cobamide analysis, 1.3 mM arabinose was added. Cultures prepared for cobamide analysis contained 1,2-propanediol (5 mM), cobyric acid (38 nM), and adenine (300 μM). Growth studies were performed at 37°C in duplicate in a 96-well microtiter plate with a computer-controlled BioTek ELx808-1 ultramicroplate reader 132 (BioTek Instruments, Inc.). Cell density analysis was performed by measuring the OD at 630 nm every 15 min for 48 h. Inoculation was done by adding 2 μl of an overnight culture grown in lysogenic broth (LB) to 198 μl of fresh medium.

Strain and plasmid construction. The *S. enterica* mutant strains (Table 1) generated in this study are variants of *S. enterica* strain JE7088. The plasmid pBAD24+*SMUL_1544* was constructed as follows. Using genomic DNA of *S. multivorans* as the template, a PCR fragment which covered the coding sequence of the *SMUL_1544* gene was produced. The sequences of the oligonucleotides (forward primer, 5′-AGCGTCATGATTGATACAATGAATGCACG-3′; reverse primer, 5′-TCAGAAGCTTTCACTTACTATCCTTTAGTATGC-3′) contained NcoI and HindIII restriction sites. Plasmid pBAD24+*SMUL_1544* was obtained after ligation of the cut PCR fragment into vector pBAD24 (21) cut with the same enzymes. Plasmid pBAD24+*SMUL_1544* was stored in *Escherichia coli* DH5α grown on LB containing 100 μg/ml ampicillin. Finally, plasmid pBAD24+*SMUL_1544* was transferred into *S. enterica* JE12941 via electroporation. Strain JE7088 and its derivatives cannot utilize arabinose as the carbon and energy source. Gene expression from the pBAD24 vector was induced with L-(+)-arabinose.

Cobamide extraction and analysis. *S. enterica* strains JE11685 and JE20971 were cultivated as described above. Cultures were grown in 2-liter Erlenmeyer flasks containing 1 liter of medium. After a 3-day incubation period at 37°C (shaking at 100 rpm), cells were harvested by centrifugation at 4°C using an Avanti J-25I centrifuge equipped with a JA25.50 rotor; cell pellets were stored at −20°C until they were used. Cobamide extraction was conducted in accordance with the method described elsewhere (22). For cobamide extraction, *S. multivorans* was grown on pyruvate- and fumarate-containing medium. The procedure for cobamide extraction and analysis via high-performance liquid chromatography (HPLC) is described elsewhere (23). High-resolution mass spectra were recorded using a Bruker Maxis high-resolution quantitative time of flight mass spectrometer equipped with an electrospray ionization source operated in the positive mode (Bruker Daltonik GmbH, Bremen, Germany). HPLC separations were achieved using a Dionex UltiMate 3000 instrument equipped with a Phenomenex Gemini C_{18} column (250 by 2 mm, 5 μm) operated at a flow rate of 400 μl/min starting with 3% aqueous acetonitrile for 5 min, followed by a linear gradient to 100% acetonitrile within 35 min, using 0.5% acetic acid as an additive. PceA activity measurement and immunological detection of the enzyme in crude cell extracts of *S. multivorans* cells were performed as previously described (23).

Isotopic labeling with L-[3-^{13}C]serine and NMR spectroscopy. *S. multivorans* was grown and subcultured in medium containing PCE as the electron acceptor in the presence of 1 mM L-Ser. The third culture contained 1 mM L-[3-^{13}C]serine. After harvesting of the cells, cobamide extraction was performed as described above. Norpseudo-B_{12} (20 μg) was isolated by semipreparative HPLC using an Agilent HP-1100 HPLC instrument equipped with a Grom-Sil 120 ODS-4 HE column (250 by 8 mm; particle size, 5 μm) and UV detector at a λ of 360 nm and coupled to a Gilson 206 Abimed fraction collector. A flow rate of 2 ml/min was used, starting at 3% acetonitrile in 0.1% aqueous acetic acid for 3 min, followed by a linear increase to 100% acetonitrile with 0.1% acetic acid over 30 min. Nuclear magnetic resonance (NMR) spectra were recorded in D_2O on a Bruker Avance III HD700 NMR spectrometer (Bruker BioSpin, Karlsruhe, Germany) at operating frequencies of 700 MHz for ^{1}H and 176 MHz for ^{13}C. A triple-resonance TCI 1.7-mm MicroCryoProbe was used to measure the spectra at 298 K.

RESULTS

Complementation of a *Salmonella enterica* Δ*cobD* strain. A characterization of the *SMUL_1544* gene function in *S. multivorans* has not been possible due to the lack of the genetic tools needed to inactivate the genes of interest in this organism. Therefore, heterologous expression of the gene encoding SMUL_1544 in appropriate *S. enterica* mutant strains offers a viable option for the testing of enzyme function. *S. enterica* strain JE7088 (Table 1) lacks the gene encoding the cobamide-independent methionine synthase (MetE). Methionine biosynthesis in this strain requires a functional cobamide-dependent methionine synthase (MetH). Hence, growth of the strain is strictly dependent on the availability of methionine or cobamides. The latter can be provided either by *de novo* biosynthesis or exogenously. The wild-type allele of *SMUL_1544* cloned under the control of the P_{araBAD} promoter was used in the experiments described below. Three derivatives of strain JE7088 were analyzed. Strain JE11685, which carried the empty cloning vector, served as a positive control. Strain JE14935, which harbored the empty cloning vector and a chromosomal deletion of the *cobD* gene, served as the negative control. Strain JE20971 harbored a *cobD* deletion and a plasmid containing the *SMUL_1544* gene. The strains used either did not make CobD or produced *S. enterica* CobD or SMUL_1544. We monitored the growth of the above-described strains under aerobic conditions in minimal medium supplemented with either dicyano-cobinamide [$(CN)_2$Cbi] or dicyano-cobyric acid [(CN_2)Cby] (9, 24). As shown in Fig. 2, none of the mutant strains grew in the absence of these additives (circles). This effect was reversed in all cultures

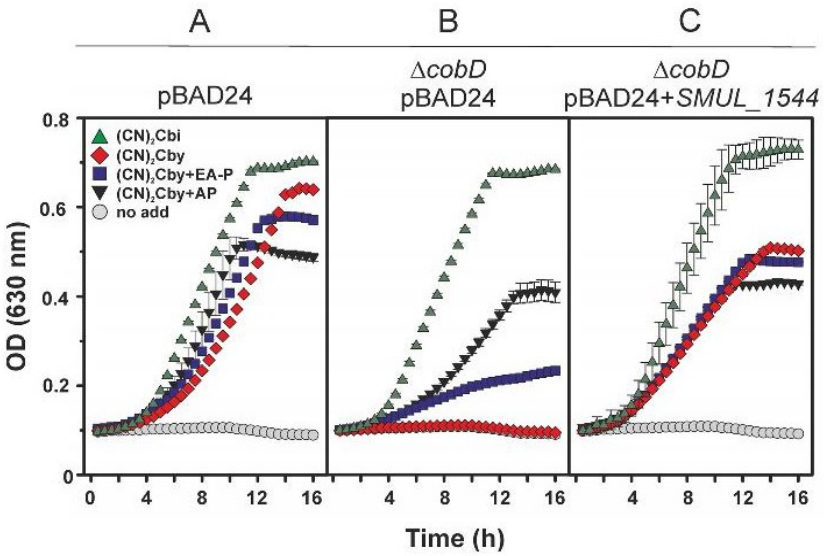

FIG 2 Cobamide-dependent growth of *S. enterica* mutant strains. (A) Strain JE11685/pBAD24; (B) strain JE14935 Δ*cobD*/pBAD24; (C) strain JE20971 Δ*cobD*/pBAD24+*SMUL_1544*. Cultivation was conducted in the presence of either dicyanocobinamide [$(CN)_2$Cbi; 1 nM], dicyanocobyric acid [$(CN)_2$Cby; 1 nM], $(CN)_2$Cby and 1-aminopropan-2-ol (AP; 1 mM), $(CN)_2$Cby, or ethanolamine phosphate (EA-P; 1 mM) or without any amendment (no add, no addition).

when 1 nM vitamin B_{12} (Coβ-cyano-Coα-5,6-dimethylbenzimidazolyl-cobamide) was added to the medium (data not shown). The addition of vitamin B_{12} to the medium had the same effect on the growth rate and yield for all the strains tested. A similar result but with a lower rate and a lower yield (reduction of about 50%) was obtained when 1 nM $(CN)_2$Cbi was present in the medium (Fig. 2, green triangles). As expected, when $(CN)_2$Cbi was used, the CobD function was not required, since $(CN)_2$Cbi already contains the linker moiety. When 1 nM (CN_2)Cby was added to the cultures, the presence of a functional CobD was necessary, as seen by the growth defect of strain JE14935, which lacks the *cobD* gene (Fig. 2B, diamonds). This defect was partially reversed by the addition of either 1 mM AP (Fig. 2B, black triangles) or 1 mM EA-P (Fig. 2B, squares). *S. enterica* was previously shown to be able to use exogenous AP and EA-P for cobamide biosynthesis (8, 9, 12). The correction of the growth defect by (CN_2)Cby plus EA-P was not as efficient as that for (CN_2)Cby plus AP, which suggested a preference of MetH for cobamides rather than norcobamides. A growth defect in the presence of (CN_2)Cby was not detectable for strain JE11685, which had a functional *cobD* gene in its genome, and for strain JE20971, which produced SMUL_1544 exclusively from the plasmid (Fig. 2A and C, diamonds). The latter observation clearly shows that SMUL_1544 can compensate for the absence of the CobD function in *S. enterica*. The growth of strains JE11685 (in which *cobD* is encoded by the genome) and JE20971 (in which *SMUL_1544* is carried by a plasmid) in the presence of (CN_2)Cby and the addition of either AP or EA-P (Fig. 2A and C, black triangles and squares, respectively) did not result in the recovery of the growth rate and yield achieved with (CN_2)Cbi supplementation (Fig. 2A and C, green triangles). The latter observation might be explained by the additional efforts that the cells have to make in taking up EA-P or AP. Phosphorylation of AP is required prior to its use in cobamide linker biosynthesis (9).

To identify the type of cobamide generated by the SMUL_1544-producing strain (JE20971), cobamide extraction and analysis were performed (Fig. 3). The cobamide extract was analyzed via HPLC, and the profile was compared to that for a cobamide extract from strain JE11685, which synthesized the native *S. enterica* CobD enzyme. Cells were grown aerobically on glycerol-containing medium supplemented with $(CN)_2$Cby. Adenine (300 μM) was added to the medium in order to sustain cobamide production. From the comparison with the HPLC elution profile of the pseudovitamin B_{12} standard, it was concluded that strain JE11685 produced pseudo-B_{12} under these growth conditions. In contrast, the SMUL_1544-producing strain JE20971 appeared to form mainly norpseudo-B_{12}. No change in the HPLC profile was observed when 1 mM L-Ser-P was added to the growth medium of JE20971. L-Ser-P is the putative substrate of SMUL_1544. Surprisingly, strain JE20971 produced both pseudo-B_{12} and norpseudo-B_{12} when 1 mM L-Thr-P was present in the growth medium. This observation suggested that SMUL_1544 might be able to use L-Ser-P and L-Thr-P as the substrates to yield EA-P and AP-P, respectively.

Production of pseudo-B_{12} in *S. multivorans*. The evidence for the conversion of L-Thr-P by SMUL_1544 obtained by the previous experiment raised the question of pseudo-B_{12} formation in *S. multivorans*. Guided cobamide biosynthesis was applied to test the organism for this synthetic capability. Cobamides from cells grown in the presence of 1 mM L-Thr-P were purified and analyzed via HPLC (Fig. 4). A cobamide which, in comparison to the elution profile of norpseudo-B_{12}, showed a peak at a higher retention time in the HPLC analysis was obtained. Using liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, the cobamide was identified as pseudovitamin B_{12} on the basis of the single charged molecular ion signal at *m/z* 1,345.5489 $[M+H]^+$ (calculated for $C_{59}H_{85}CoN_{17}O_{14}P$, *m/z* 1,345.5526; difference, −2.7 ppm) and the double charged molecular ion signal

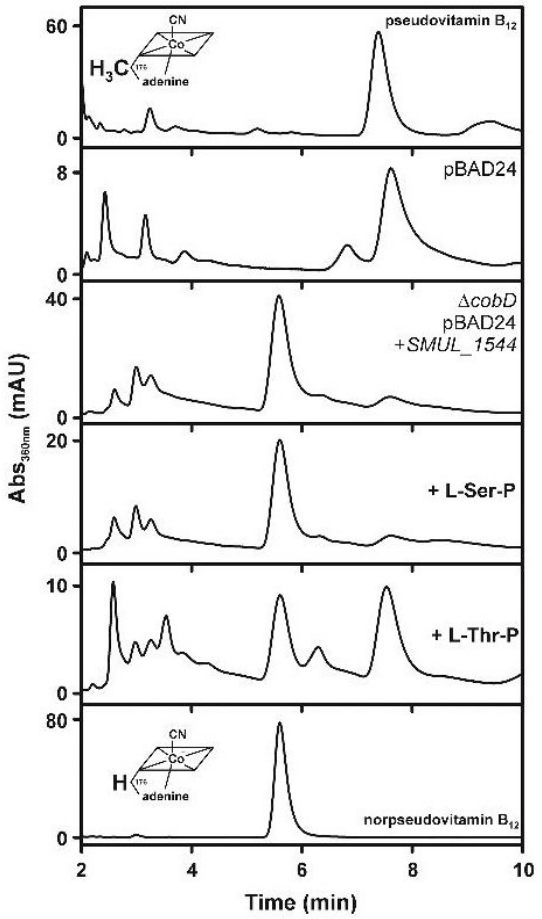

FIG 3 HPLC analysis of cobamide extracts from *S. enterica* JE7088 mutant strains. Strain JE11685/pBAD24 and strain JE20971 Δ*cobD*/pBAD24+SMUL_1544 were analyzed. L-Ser-P or L-Thr-P was added to the culture of JE20971 at a concentration of 1 mM. Pseudo-B_{12} extracted from *Propionibacterium acidipropionici* and norpseudo-B_{12} extracted from *S. multivorans* served as standards. The suffix vitamin refers to the presence of a cyano group as the upper ligand in the extracted cobamides. mAU, milliabsorbance units.

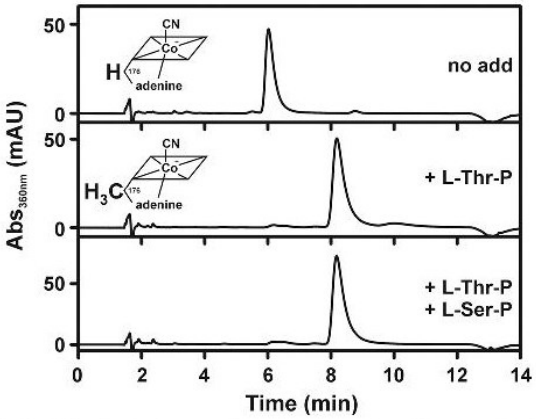

FIG 4 HPLC analysis of cobamide extracts from *S. multivorans*. L-Ser-P or L-Thr-P was added to the medium to a final concentration of 1 mM.

at *m/z* 673.2801 $[M+2H]^{2+}$ (calculated for $C_{59}H_{86}CoN_{17}O_{14}P$, *m/z* 673.2799; difference, −0.3 ppm). Confirmation of the LC-MS/MS data was obtained with an authentic standard isolated from *Propionibacterium acidipropionici*. The addition of 1 mM L-Ser-P did not counteract the effect of L-Thr-P.

The presence of the methyl group at position 176 in the linker moiety of pseudo-B_{12} might have a negative impact on the binding of the cofactor to the PceA enzyme in *S. multivorans*. Such an effect might be observable from a lower growth rate or a lower yield of the organism with PCE as the sole electron acceptor. For this reason, we analyzed the growth kinetics of the organism (Fig. 5A). Two subsequent precultures were grown under conditions identical to those of the growth experiment. The inoculum (10%, vol/vol) was transferred after 24 h when the culture without additives reached stationary phase. The growth rate and yield of the cultures with L-Thr-P (1 mM) were not drastically altered in comparison to those of the cultures without supplementation. However, growth started after an extended lag phase. For the culture without an amendment, a maximal growth rate of 0.059 h^{-1} was calculated. This value was similar to the value obtained for the culture treated with L-Thr (0.060 h^{-1}). The maximal growth rate of the cells cultivated in the presence of L-Thr-P was 0.041 h^{-1}. No effect was observed when L-Thr rather than L-Thr-P was in the medium. The effect of L-Thr-P was specific for the PCE-dependent growth of *S. multivorans*, since cells cultivated on fumarate rather than PCE as the electron acceptor were not affected by the presence of L-Thr-P (data not shown).

The PceA enzyme activity was measured in crude extracts of PCE-grown cells cultivated either with or without L-Thr or L-Thr-P (Fig. 5B). For all conditions applied in this experiment, the cells were harvested in the late exponential growth phase (protein concentration, 70 to 80 μg/ml). The presence of L-Thr had no impact on the conversion of PCE by PceA in the photometric enzyme assay. In crude extracts obtained from cells grown in the presence of L-Thr-P, a 50% reduction in the PceA activity was measured. This result does not exactly reflect the impact of L-Thr-P on growth in the respective culture (reduction of μ_{max} by about 30%) and might point toward a surplus of PceA in *S. multivorans* cells, the activity of which is not linked to energy conservation and growth. The proteins in the crude cell extracts were separated by SDS-PAGE and analyzed by Western blot analysis using polyclonal PceA antibodies (Fig. 5C). As it was shown before, two forms of the PceA enzyme were detected. The slower-migrating band represented the Tat signal peptide-bearing precursor (prePceA), and the faster-migrating band represented the mature form of the enzyme without the signal peptide (PceA). In cells grown in the absence or presence of L-Thr, the mature form of PceA was the dominant species. In cells cultivated in medium supplemented with L-Thr-P, the overall amount of PceA was slightly reduced, which suggested a decrease in the stability of the enzyme.

Isotopic labeling of the linker moiety norpseudo-B_{12}. Cobamide linker biosynthesis in *S. multivorans* can be easily redirected by the addition of L-Thr-P to the growth medium, which points toward a biosynthetic pathway comparable to what was reported for *S. enterica* (Fig. 1). For a better understanding of the synthesis

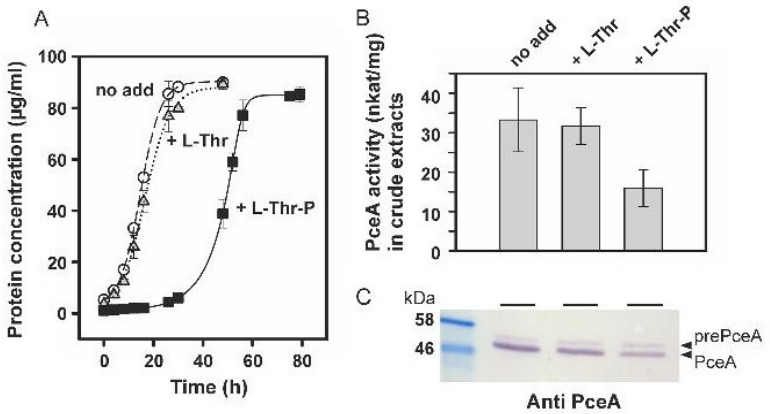

FIG 5 PCE-dependent growth and PceA activity in *S. multivorans*. (A) Growth on pyruvate- and PCE-containing medium; (B) PceA enzyme activity in crude extracts; (C) immunological detection of PceA in crude extracts separated via SDS-PAGE. L-Thr or L-Thr-P was added to the medium at a final concentration of 1 mM.

of EA-P, it is important to determine its metabolic origin. L-Ser-P is produced as an intermediate in L-Ser biosynthesis from 3-phosphoglycerate in *S. multivorans*. This was inferred from the analysis of the organism's genome. In analogy to the linker biosynthetic pathway in *S. enterica*, where L-Thr is phosphorylated by the specific kinase (PduX) and used as the linker precursor, L-Ser might be used in a similar manner by *S. multivorans*. A PduX-like protein was not encoded by the norcobamide biosynthesis gene cluster of *S. multivorans*, nor was a gene encoding a putative kinase identified among the 25 open reading frames containing most of the cobamide biosynthetic genes of this bacterium. To analyze the ability of *S. multivorans* to utilize L-Ser as a precursor for the linker moiety in norpseudo-B_{12}, an isotopic labeling approach was conducted using L-[3-^{13}C]serine. NMR analysis of a purified norpseudo-B_{12} sample from *S. multivorans* grown in the presence of 1 mM L-[3-^{13}C]Ser revealed the ^{13}C incorporation into position 176 in the linker moiety (Fig. 6), demonstrating that this unit was derived from L-Ser. These data reveal the possibility of the formation of EA-P via L-Ser-P from serine and underpin the previous observation that, besides L-Thr-P, L-Ser-P might also be a substrate of SMUL_1544. Hence, this result indicated the utilization of L-Ser-P as a precursor of the EA-P linker moiety in norpseudo-B_{12} and makes the use of other putative intracellular sources of EA-P (e.g., phospholipids) rather unlikely. Incorporation of ^{13}C from L-[3-^{13}C]Ser into seven methyl groups of the corrin ring was also observed. The same methyl units are known to be labeled by [*S*-$^{13}CH_3$]methionine ([*S*-$^{13}CH_3$]Met) (25), which is converted into *S*-adenosylmethionine (SAM), the methyl donor used by cobamide biosynthetic enzymes. The formation of Met from homocysteine is catalyzed by a cobamide-independent methionine synthase (MetE) in *S. multivorans* (16). Methyltetrahydrofolate serves as the donor of the methyl group transferred in this reaction (26). Tetrahydrofolate can be methylated at the expense of Ser in *S. multivorans*, which channels the ^{13}C label of L-[3-^{13}C]-Ser into Met, SAM, and, finally, the methyl groups of the corrin ring (27).

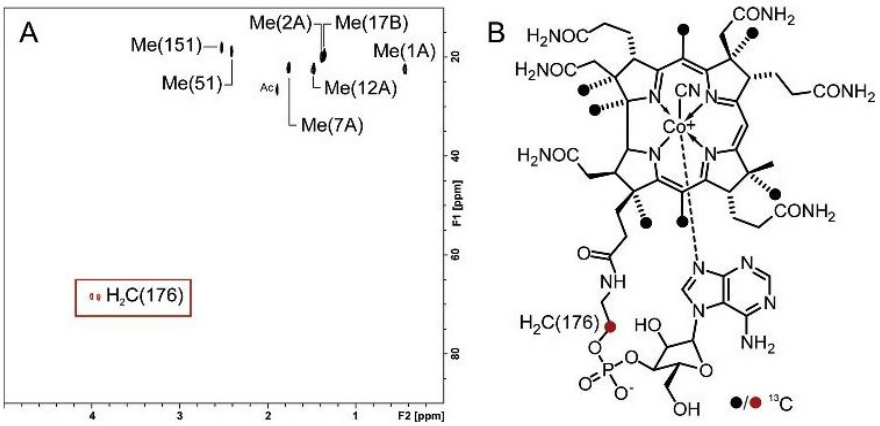

FIG 6 Incorporation of L-[3-^{13}C]Ser into the linker moiety in norpseudo-B_{12}. (A) Partial heteronuclear single quantum coherence spectrum of norpseudo-B_{12} after feeding with L-[3-^{13}C]serine shows peaks corresponding to seven SAM-derived methyl groups of the corrin ring as well as the methylene group at position 176 [$H_2C(176)$] of the linker moiety (the peak assignments are according to previous work [6]). (B) Structure of norpseudo-B_{12}. ^{13}C-labeled positions are indicated by filled circles. Me, methyl group; Ac, acetic acid.

DISCUSSION

The CobD-mediated formation of AP-P as a precursor of the linker moiety is one of the last steps in the formation of cobamides. The absence of this enzyme arrests the cobamide-dependent growth of *S. enterica*. Herein we showed that expression of the gene *SMUL_1544* from *S. multivorans* (Fig. 2) complemented this effect. From this result, it could be concluded that the gene product SMUL_1544 functions as an L-Thr-P decarboxylase. However, when SMUL_1544 was produced in *S. enterica*, this bacterium synthesized norpseudo-B_{12} rather than pseudo-B_{12} (Fig. 3). This finding pointed toward the formation of EA-P rather than AP-P and suggested that SMUL_1544 prefers L-Ser-P over L-Thr-P as the substrate. L-Thr-P is specifically formed for cobamide biosynthesis in *S. enterica*, and L-Ser-P is an intermediate of the common serine biosynthetic pathway from 3-phosphoglycerate. Hence, both compounds should be available in *S. enterica* cells. Excess L-Thr-P in the medium led to the production of pseudo-B_{12} by the SMUL_1544-producing strain (Fig. 3). L-Thr-P is taken up by the cells in an amount that appears to suppress the use of L-Ser-P by SMUL_1544. These observations led to the conclusion that SMUL_1544 is a decarboxylase that can use L-Ser-P or L-Thr-P as the substrate, but with a preference for L-Ser-P. Direct proof of the conversion of L-Ser-P and L-Thr-P by SMUL_1544 has to await the purification and characterization of the protein. The decarboxylation of L-Ser-P has not been investigated for *S. enterica* CobD so far but is also going to be assessed in future studies.

The hypothesis that SMUL_1544 can decarboxylate both L-Thr-P and L-Ser-P was tested in the PCE-respiring bacterium *S. multivorans*. L-Ser-P is expected to be available in *S. multivorans*, since it is an intermediate of the serine biosynthetic pathway. The organism lacks a specific L-Thr kinase (PduX), which makes the availability of intracellular L-Thr-P unlikely. The formation of pseudo-B_{12} was exclusively observed when the organism was cultivated in the presence of L-Thr-P (Fig. 4). This finding revealed the efficient uptake of the phosphorylated amino acid in this bacterium. In *S. enterica* and *S. multivorans*, the effect of L-Thr-P was not reversed by the addition of L-Ser-P at the same concentration. A rate of L-Ser-P uptake lower than the rate of L-Thr-P uptake might be the reason for this result. An excess of L-Thr-P in the cells could overcome the preferential use of L-Ser-P by SMUL_1544. The utilization of L-Ser-P by SMUL_1544 in its native host was deduced from the incorporation of exogenous ^{13}C-labeled serine into the norpseudo-B_{12} cofactor produced by *S. multivorans* (Fig. 6). At present, it is unclear whether or not a serine kinase is specifically functioning in norpseudo-B_{12} biosynthesis in *S. multivorans*. Results from our ^{13}C-NMR experiments (Fig. 6) suggest that this may be the case.

The identification of the norpseudo-B_{12} cofactor in *S. multivorans* more than 10 years ago raised the question of a specific need for this unusual type of cobamide in the organism. In the study presented here, it was shown that the formation of pseudo-B_{12} in *S. multivorans* does not hinder the maturation of the cobamide-containing PceA enzyme (Fig. 5). A negative effect on the maturation of the enzyme may be concluded from the decrease in PceA enzyme activity and the slight reduction in the amount of PceA in cells forming pseudo-B_{12}. However, besides an extension in the lag phase of a pseudo-B_{12}-producing culture growing with PCE, no severe effect on the growth rate or yield was detected. The almost exclusive production of pseudo-B_{12} concomitantly with the formation of active PceA enzyme makes the binding of this cofactor to the enzyme's precursor in the cytoplasm very likely. Hence, the PceA structure might adopt a conformation which allows the binding of either norpseudo-B_{12} or pseudo-B_{12}. Such an acceptance of a structurally different cobamide cofactor by PceA has not been observed before, when the lower base of the nucleotide loop in norpseudo-B_{12} was exchanged from adenine to 5,6-dimethylbenzimidazole (23). Current work in our laboratory seeks to determine whether or not catalytically active PceA from cells cultivated in the presence of L-Thr-P contains pseudo-B_{12} or whether it selectively binds traces of norpseudo-B_{12}.

The production of norcobamides has not yet been reported for B_{12}-synthesizing prokaryotes different from *S. multivorans*. Furthermore, a gene product with high similarity to SMUL_1544 and with a defined role in cobamide biosynthesis has not been identified so far. The firmicute *Ilyobacter polytropus*, which harbors the cobamide biosynthesis genes with the highest similarity to those of *S. multivorans*, was shown to produce pseudo-B_{12} (16). The sequence identity of CobD from *I. polytropus* and SMUL_1544 is exceptionally low compared to that of the other cobamide biosynthesis genes. The low sequence similarity toward CobD homologues in general and the special function of SMUL_1544 in norcobamide biosynthesis make this enzyme occupy an exceptional position among the PLP-dependent decarboxylases.

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2.4 Functional analysis of a unique L-serine *O*-phosphat*e* decarboxylase essential for the norcobamide biosynthesis in *Sulfurospirillum multivorans*

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In this study purified SMUL_1544 was identified as ethanolamine *O*-phosphate (EA-P) forming L-serine *O*-phopshate (L-Ser-P) decarboxylase that is additionally able to decarboxylate L-threonine *O*-3-phosphate (L-Thr-P) with a lower conversion rate. A sequence alignment of SMUL_1544 with *Salmonella enterica* CobD (*Se*CobD) that preferably decarboxylates L-Thr-P and mutagenesis revealed that the unique Ser242, which is replaced by a highly conserved threonine in CobD homologs is important for the L-Ser-P decarboxylation by the *S. multivorans* enzyme. SMUL_1544 had no aminotransferase activity with L-histidinol phosphate (L-His-C), but showed a closer relatedness to L-His-P aminotransferases than to CobD enzymes in a phylogenetic analysis indicating SMUL_1544 as a putative evolutionary link between both classes of enzymes.

Contribution of Sebastian Keller to this study: 90 %

Sebastian Keller performed all experiments including the bioinformatics and he mainly wrote the manuscript.

Functional analysis of a unique L-serine *O*-phosphat*e* decarboxylase essential for the norcobamide biosynthesis in *Sulfurospirillum multivorans*

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SUMMARY

Structural diversity of natural cobamides *de novo* synthesized by certain prokaryotes is based on the presence of alternative lower bases in the nucleotide loop. This ribosyl bound lower ligand is connected to the D ring of the corrin center via an (*R*)-1-aminopropan-2-ol *O*-2-phosphate (AP-P) linker in almost all known natural cobamides. The AP-P is produced by the L-threonine *O*-3-phosphate (L-Thr-P) decarboxylase CobD. In this study, the CobD homolog of the organohalide-respiring *Sulfurospirillum multivorans*, SMUL_1544, was purified and characterized as an L-serine *O*-phosphate (L-Ser-P) decarboxylase synthesizing ethanolamine *O*-phosphate (EA-P), the unique linker of norcobamides that are exclusively produced by this organism. A comparative analysis of SMUL_1544 and *Se*CobD, the L-Thr-P decarboxylase of *Salmonella enterica*, uncovered the decarboxylation capability of L-Thr-P and L-Ser-P by both enzymes. However, while *Se*CobD is specialized on L-Thr-P, SMUL_1544 preferably converted L-Ser-P. The presence of a unique serine residue (Ser242) in SMUL_1544 was shown to be crucial for the L-Ser-P decarboxylation. The exchange of Ser242 by a threonine, highly

conserved in L-Thr-P decarboxylases such as *Se*CobD reversed the substrate preference to some extent. SMUL_1544 is a pyridoxal-5'-phosphate (PLP) containing enzyme, which is phylogenetically positioned between PLP-dependent L-Thr-P decarboxylases and aminotransferases. However SMUL_1544 displayed no aminotransferase activity. Its phylogenetic position and unique substrate preference make SMUL_1544, the CobD of *S. multivorans*, and interesting subject for studying enzyme evolution.

INTRODUCTION

Cobamides are essential cofactors of different enzymes in prokaryotes, lower eukaryotes, and animals (Escalante-Semerena and Warren 2008). These macrocyclic cofactors consist of a conserved terapyrrolic corrin ring and variable axial ligands of the central cobalt ion (Renz 1999). Cobamides are exclusively biosynthesized *de novo* by a subset of prokaryotic species in a more than 25 enzymes involving pathway (Renz 1999, Warren *et al.* 2002). The most variable structure of these natural cobamides is the lower axial ligand, which is mostly a purine or a benzimidazole derivative (Renz 1999). The lower base is part of a nucleotide loop including a linker molecule that connects the corrin ring with the ribosyl-bound base. All but one prokaryotes synthesize cobamides with an (*R*)-1-aminopropan-2-ol *O*-2-phosphate (AP-P) linker (Warren *et al.* 2002). The organohalide respiring ε-proteobacterium *Sulfurospirillum multivorans* produces the unusual cobamide norpseudo-B_{12} containing a unique ethanolamine *O*-phosphate (EA-P) linker with a methyl group less than AP-P and an adenine moiety as lower ligand (Scholz-Muramatsu *et al.* 1995, Kräutler *et al*. 2003). It serves as cofactor of the tetrachloroethene reductive dehalogenase PceA that reductively dechlorinates tetrachloroethene (PCE) to *cis*-1,2-dichloroethene via trichloroethene (Neumann *et al.* 1995, Bommer *et al.* 2014). The biosynthesis of the common AP-P linker and its attachment to the corrin ring was first described for *Salmonella enterica* subsp*. enterica* serovar *Thyphimurium* strain LT2 (henceforth termed *S. enterica*). Three enzymes were found to be involved in this pathway (Brushaber *et al.* 1998, Zayas *et al*. 2007, Fan and Bobik 2008). The amino acid L-threonine is phosphorylated to L-threonine *O*-3-phosphate (L-Thr-P) by the L-threonine kinase PduX (EC 2.7.1.177). Subsequently the carboxyl group of L-Thr-P is removed by the activity of the L-threonine *O*-3-phosphate decarboxylase CobD (EC 4.1.1.81) yielding AP-P, which is attached to the corrin ring compound adenosylcobyric acid by the adenosylcobinamide

phosphate synthase CbiB (EC 6.3.1.10). However, less was known about the EA-P linker biosynthesis in *S. multivorans*. This microaerophilic bacterium contains a complete set of genes for the anaerobic biosynthesis of norpseudo-B_{12} in its genome (Goris *et al*. 2014). Two genes encoding for CbiB and a putative CobD homolog (SMUL_1544) were found inside this contiguous cobamide biosynthesis gene cluster. Previous studies with heterologously expressed *SMUL_1544* in a *cobD* and *metE* (B_{12}-independent methionine synthase, EC 2.1.1.14) deletion strain of *S. enterica*, dependent on the activity of the cobamide-harboring methionine synthase MetH (EC 2.1.1.13, Rowbury 1983, Saint-Girons *et al*. 1988), revealed that SMUL_1544 complemented the *cobD* growth defect on glycerol minimal medium (Keller *et al*. 2016). Moreover, the EA-P linker harboring norpseudo-B_{12} was the predominantly produced cobamide by *S. enterica* Δ*cobD* in the presence of active SMUL_1544. This result pointed towards a key role of this enzyme in the EA-P linker biosynthesis in *S. multivorans*. Interestingly when 1 mM L-Thr-P, the physiological substrate of *S. enterica* CobD (*Se*CobD), was exogenously applied to *S. multivorans* cultures, a biosynthesis of AP-P rather than EA-P containing pseudo-B_{12} was observed. This finding indicated that SMUL_1544 is able to convert not only its physiological substrate to EA-P, but probably also L-Thr-P to AP-P. Additionally these observations affirmed the hypothesis that SMUL_1544 is a decarboxylase involved in cobamide biosynthesis in *S. multivorans*. The observation that AP-P and EA-P cobamides were produced by one organism proofed CbiB of *S. multivorans* to be not restricted in the incorporation of one specific linker moiety into the cobamide, a feature it shares with *S. enterica* CbiB (Zayas *et al.* 2007). The physiological substrate of SMUL_1544 was not identified yet, but L-serine *O*-phosphate (L-Ser-P) was assumed, which would explain the lack of the methyl group in the linker of norpseudo-B_{12}.

The common cofactor of SMUL_1544 and *Se*CobD enzymes is pyridoxal-5'-phosphate (PLP), the biological active form of vitamin B_6 (Brushaber *et al*. 1998, Cheong *et al*. 2002a). This cofactor was found in 160 different enzymes, which belong to diverse essential pathways in all vital organisms (Percudani and Peracchi 2003). The PLP dependent enzymes catalyze different reactions including decarboxylations, transaminations, racemizations, β- and γ-elimination reactions. The cofactor is bound to a lysine residue inside the protein via a Schiff base linkage (internal aldimine) in almost all PLP-dependent enzymes prior to and after catalysis (John 1995, Eliot and Kirsch 2004, Cook *et al.* 2006). Based on the structural

organization of the PLP-enzymes five superfamilies with each several subfamilies were divided. *Se*CobD as well as SMUL_1544 belong to the aspartate aminotransferase (fold I) superfamily and their PLP binding site sequences are typical for the aminotransferase class 1/class 2 type subfamily (Pfam: PF00155, Grishin *et al*. 1995, Sigrist *et al*. 2013). The enzyme L-histidinol phosphate aminotransferase HisC (EC 2.6.1.9) belongs to the same family and was already shown to have a sequence similarity to *Se*CobD (Brushaber *et al.* 1998, Cheong *et al*. 2002b). These enzymes transfer the α-amino group of L-histidinol phosphate (L-His-P) in a first reaction to PLP forming 3-(imidazole-4-yl)-2-oxopropyl phosphate and pyridoxamine-5'-phosphate (PMP). This amino group of PMP is then transferred to 2-oxoglutatrate releasing L-glutamate and the original cofactor PLP is restored (Mizuguchi *et al*. 2003). However, *Se*CobD showed no aminotransferase activity of any amino acid tested *in vitro* (Brushaber *et al.* 1998). The putative CobD protein SMUL_1544 is with 392 amino acids (aa) 28 residues longer than *Se*CobD. Based on basic local alignment (blastp, Altschul *et al.* 1990), SMUL_1544 revealed the highest similarities to the annotated, but yet uncharacterized L-His-P/aromatic aminotransferases or cobyric acid decarboxylases of the ε-proteobacteria *Campylobacter jejuni* (390 aa, WP_052797156.1) and *Arcobacter butzleri* LT354 (390 aa, KLE11288.1) (Smith and Muldoon 1974, Vandamme *et al.* 1992). This similarity to predicted aminotransferases and its previously shown role in the formation of the cobamide linker molecule EA-P in *S. multivorans* raised the question, if SMUL_1544 has a decarboxylase and an additional aminotransferase activity. In this study SMUL_ 1544 was purified and characterized towards these activities *in vitro*. The aim of this study was to unravel the physiological substrate of SMUL_1544 in the EA-P cobamide linker formation in *S. multivorans* and to compare the functionality of the enzyme with *Se*CobD. The three dimensional structure of *Se*CobD with PLP, L-Thr-P or the product aldimine-complex in the active site was previously determined and revealed several amino acids crucial for binding substrate and cofactor in the enzyme (PDB files 1lkc, 1lc7 and 1lc8, respectively, Cheong *et al*. 2002a, b). A sequence comparison of SMUL_1544 with *Se*CobD as well as with other members of the aspartate aminotransferase family of PLP-dependent enzymes was performed to investigate the evolutionary position of the *S. multivorans* protein among decarboxylases and aminotransferases.

Results

SMUL_1544 is an L-serine *O*-phosphate decarboxylase

In order to characterize SMUL_1544, the respective gene was heterologously expressed in *E. coli* and the gene product bearing a C-terminal Strep-tag was purified via affinity chromatography. For the monitoring of the SMUL_1544 enzyme activity an *in vitro* decarboxylation assay with L-Ser-P or L-Thr-P as substrates was applied. After chemical derivatization the reaction mixtures were separated via HPLC coupled to photometric detection (Fig. S1). In accordance to the assumption that L-Ser-P might be converted to EA-P by the enzyme, the purified SMUL_1544 decarboxylated catalyzed this reaction with a specific activity of 3.4 nkat/mg protein under the applied conditions (Tab. 1). Treatment of the reaction mixture with alkaline phosphatase led to the appearance of products co-eluting with L-serine and ethanolamine on HPLC (Fig. S2). Due to the previous observed incorporation of exogenous L-Thr-P into pseudo-B_{12} in *S. multivorans*, an additional decarboxylation ability of SMUL_1544 with L-Thr-P was assumed. Indeed, L-Thr-P was decarboxylated to AP-P with about 20 % of the activity as observed for L-Ser-P by SMUL_1544. As already described for *Se*CobD, SMUL_1544 did not decarboxylate the non-phosphorylated amino acids L-serine or L-threonine (data not shown). Additionally, purified *Se*CobD also converted L-Ser-P besides its physiological substrate L-Thr-P, but with a lower rate (Tab. 1).

Tab. 1: Specific enzyme activities of SMUL_1544 and *Se*CobD.

	Specific activity (nkat/mg)	
Enzyme	L-Ser-P	L-Thr-P
SMUL_1544	3.4 ± 0.7	0.7 ± 0.4
***Se*CobD**	1.6 ± 0.2	5.0 ± 0.5

As expected SMUL_1544 is the EA-P linker forming L-Ser-P decarboxylase of *S. multivorans*. These data clearly show a specificity of the decarboxylase SMUL_1544 for L-Ser-P that is absent in the standard-type *Se*CobD.

Sequence comparison of SMUL_1544 and *Se*CobD

A pair-wise sequence alignment of the CobD homologs SMUL_1544 and *Se*CobD (Sm and Se in Fig.1, respectively) was performed, which revealed a sequence identity of about 22 % between both proteins. Both proteins show a typical aminotransferase class 1/class 2 PLP attachment site (*cyan* in Fig. 1) (Pfam: PF00155, Interpro: IPR004839, Finn *et al.* 2016, Finn *et al.* 2017). Thus, the residue Lys243 of SMUL_1544 is revealed to be the analog of Lys216 in *Se*CobD and the residue forming the internal aldimine with PLP typical for PLP-dependent enzymes (DiSalvo *et al*. 2013). In a previous study the three-dimensional structure of *Se*CobD was unraveled (Cheong et al. 2002a, b). The decarboxylase is a homodimeric protein and the active site of each monomer is located inside a central cavity of the protein. The cofactor PLP and the substrate L-Thr-P are hold in place by hydrogen bonding established mainly with the phosphate groups in both compounds. The residues Tyr56, Glu85, Thr86, Ser213, Thr215, and Arg224 of *Se*CobD bind the phosphate in PLP (Fig.1, *green*) and His8, Gly9, Arg323, and Arg337 the phosphate group of L-Thr-P (Fig. 1, *yellow*). Most of these residues are also present in SMUL_1544 with a few exceptions. In the PLP binding motif Ala107 and Ser242 replace Glu85 and Thr215 of *Se*CobD, respectively. While the residue Thr215, directly upstream of the Schiff base forming Lys216, was found to be highly conserved among CobD homologs, Glu85 is frequently replaced by a threonine, serine, alanine, or asparagine residue (Interpro: IPR005860, Finn *et al.* 2017). In the case of the amino acids binding the substrate phosphate, the C-terminal arginines (Arg323 and Arg337 in *Se*CobD) are also conserved in SMUL_1544 (Arg354 and Arg368), but for the N-terminal residues His8 and Gly9 no analogous amino acids were detected in the *S. multivorans* protein by the alignment. The N-terminus at all displayed the lowest degree of sequence identity. SMUL_1544 has an extended N-terminus compared to *Se*CobD that is a 28 amino acids shorter protein (392 vs. 364 amino acids). However, the extended N-terminus of SMUL_1544 contains two histidines (His17 and His 26) and a glycine (Gly24) that might fulfil the same function like His8 and Gly9 in *Se*CobD. Two amino acids of *Se*CobD form hydrogen bridge bonds with the pyridine ring of PLP (Asp185 and Asn157) and

Asn157 additionally binds the phosphate group of L-Thr-P (Fig.1, *grey*; Cheong *et al.* 2002 a, b). These amino acids are highly conserved among CobD and HisC enzymes (Interpro: IPR005860 and IPR005961, Finn *et al.* 2017). Both residues are present in the SMUL_1544 sequence as Asn180 and Asp207.

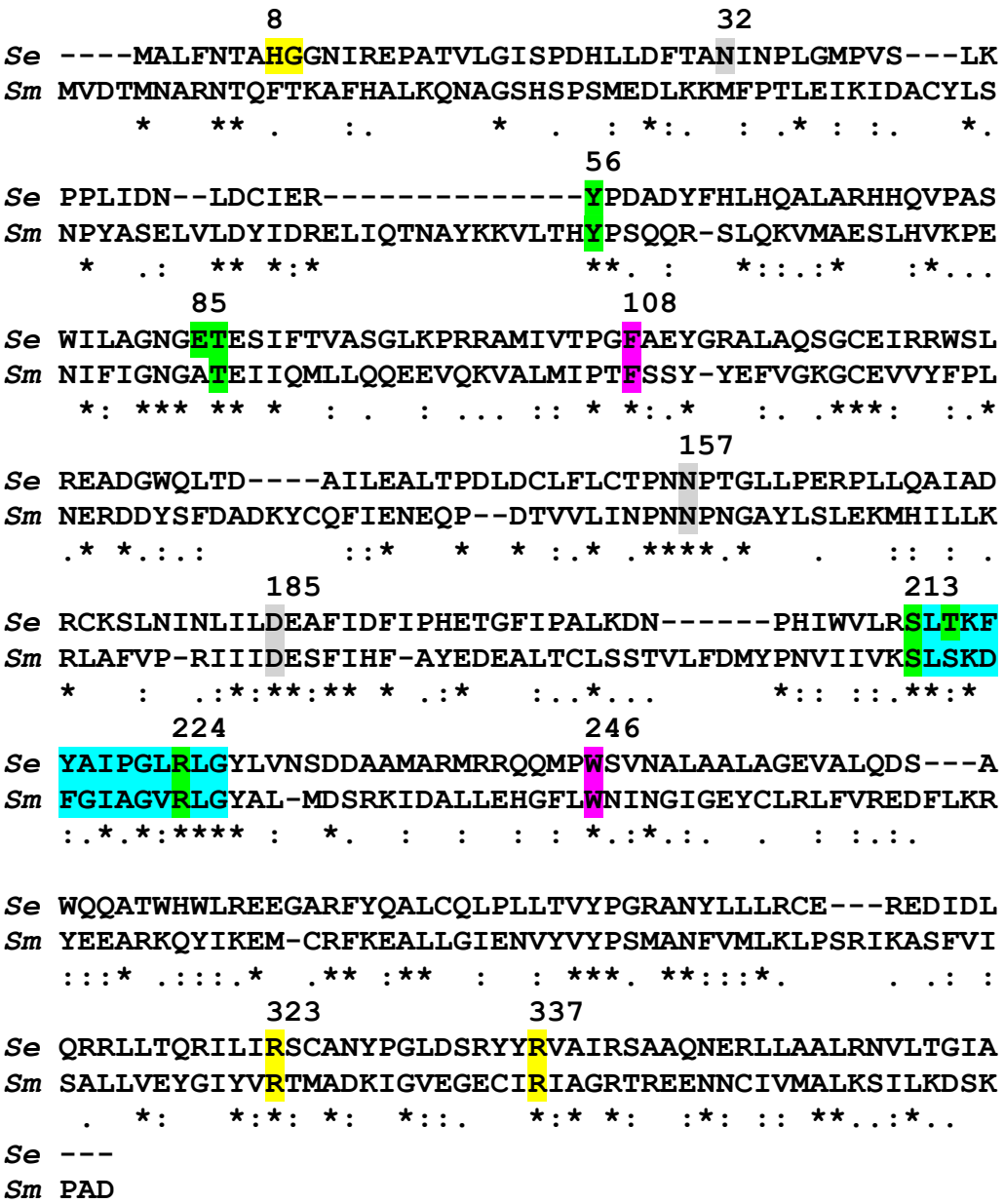

Fig. 1: Pair-wise sequence alignment of *Se*CobD (*Se*) and SMUL_1544 (*Sm*) with MUSCLE v3.8. The PLP binding site is colored in *cyan* and the residues binding the phosphate group of PLP in *Se*CobD are colored in *green*. The amino acids binding the phosphate group of L-Thr-P in *Se*CobD are highlighted in *yellow* and the other residues forming hydrogen bridge bonds with the pyridine ring of PLP are colored in *grey*. Finally the formerly identified CobD specific residues, missing in HisC proteins are highlighted in *pink*. The numbers denote the amino acid position in *Se*CobD.

Asparagin Asn32 of *Se*CobD that binds the carboxyl group of L-Thr-P with two hydrogen bridge bonds, is not conserved in SMUL_1544. Whether Asn51 has a similar function in SMUL_1544 is not known so far. When the crystal structure of *Se*CobD was solved four residues were identified in the active site cavity, which are present in L-Thr-P decarboxylases (His8, Thr86, Phe108, and Trp246), but not in the related L-histidinol phosphate aminotransferases (Cheong *et al*. 2002b). These amino acids were proposed to prevent L-His-P from binding due to clashes with the imidazole chain of L-His-P lacking in L-Thr-P. The HisC-like aminotransferase of *Escherichia coli* possesses the residues Tyr20, Asp85, Tyr110, and Tyr243 at the same positions in its active site, allowing L-His-P to bind (Haruyama *et al.* 2001). Thus, it was suggested that CobD and HisC homologs could be differentiated based on the presence of one of these sets of amino acid (Cheong *et al.* 2002b). Although His8 is absent in SMUL_1544, the other three amino acids are conserved (Thr108, Phe130, and Trp272) indicating the protein as CobD decarboxylase and not as HisC aminotransferase. Overall, SMUL_1544 contains almost all amino acids that were found to be important in *Se*CobD for the decarboxylation of L-Thr-P to AP-P.

The N-terminus of SMUL_1544

The largest sequence divergence between SMUL_1544 and *Se*CobD is found in the N-terminus of both proteins (Fig. 1). In order to understand the function of the extended N-terminus of SMUL_1544, a series of truncated proteins was generated (Fig. 2A). The smallest recombinant protein had the same number of amino acids like *Se*CobD (364 amino acids). After purification of the mutant proteins, they were analyzed with the decarboxylation assay. The conversion rate of L-Ser-P was decreased to about 50 % when 20 N-terminal amino acids were deleted (Fig. 2B). In the case of the largest deletion lacking 28 amino acids, almost no L-Ser-P conversion was detected. The L-Thr-P decarboxylation activity for all truncations, except for the smallest mutant protein (Δaa 2-29), did not change substantially compared to the wild type enzyme (Fig. 2B). These results unraveled a specific need for this last eight N-terminal amino acids (N_{22}AGSHSPS_{29}, Fig. 3A) for the functionality of the enzyme. This sequence contains a histidine (His26), which might have the same important function in binding of the substrate like His8 in *Se*CobD (Fig. 1, Cheong *et al.* 2002a, b). Therefore His26 was replaced by an alanine (H26A). The decarboxylation rate of the H26A mutant protein with L-Ser-P and L-

Thr-P were as low as those observed for SMUL_1544 lacking 28 amino acids. Hence, His26 plays a pivotal role for the decarboxylation activity of the enzyme, most probably by binding the substrate. The UV/Vis-spectroscopic analysis of the recombinant SMUL_1544 proteins H26A and Δaa 2-29 revealed both to be completely saturated with the cofactor PLP pointing towards a negative effect on the binding of the substrates L-Ser-P or L-Thr-P by these recombinant proteins.

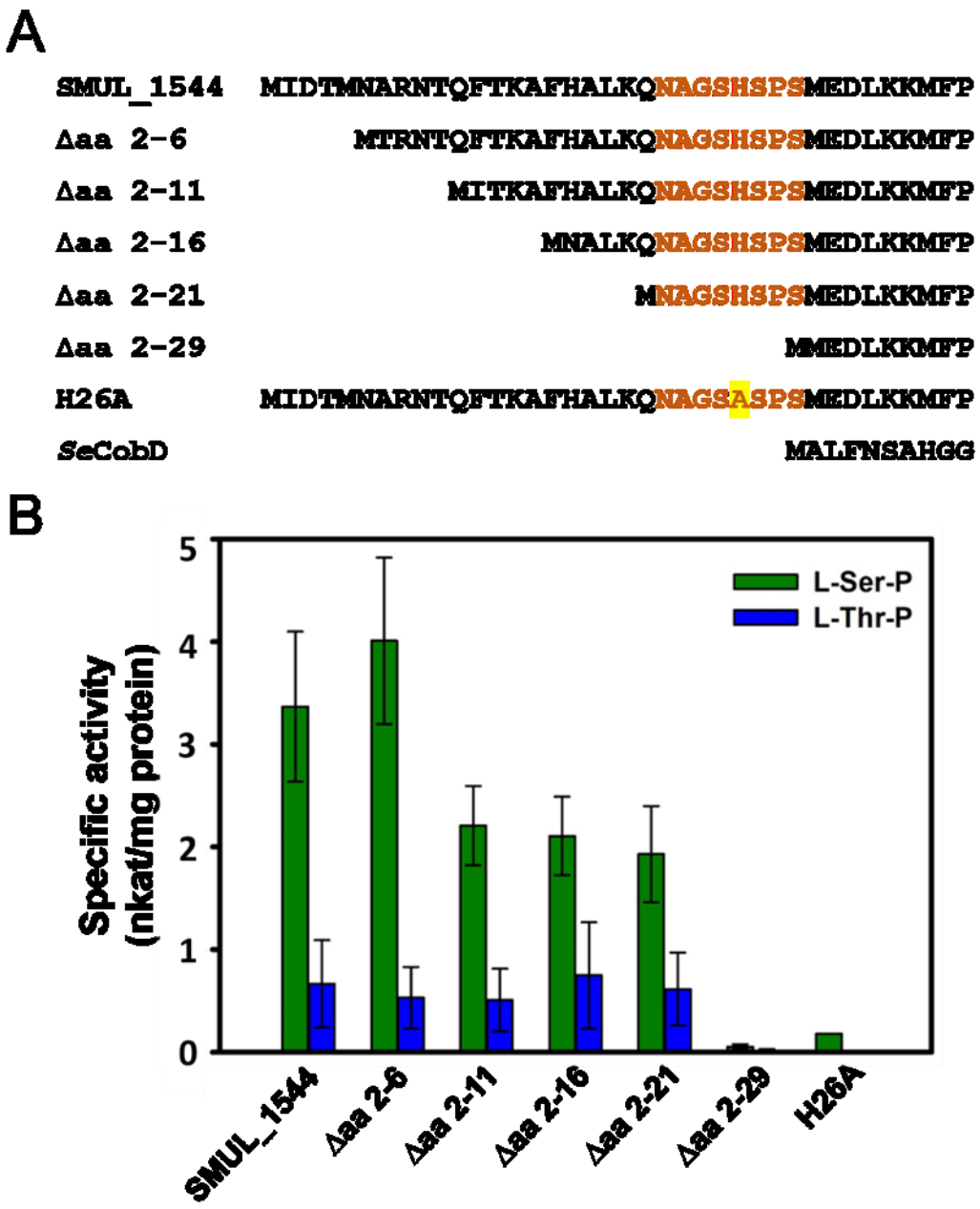

Fig. 2: Decarboxylation rates of L-Ser-P and L-Thr-P by recombinant SMUL_1544 proteins. (A) Amino acid sequences of the N-terminus of the SMUL_1544 mutant proteins and *Se*CobD. (B) Specific decarboxylation activities.

An N-terminal fusion of *Se*CobD with the first 28 amino acids of SMUL_1544 or the short sequence NAGSHSPS did not alter the decarboxylation rates of L-Ser-P and L-Thr-P (data not shown).

Amino acid Ser242 of SMUL_1545 is crucial for the substrate specificity

Two amino acids responsible for coordinating the phosphate group of PLP in *Se*CobD are altered in SMUL_1544 (Fig. 1). Glu85 is replaced by Ala107 and Thr215 by Ser42. In order to understand their impact on the substrate preference of SMUL_1544, they were separately replaced by their respective counterpart. The exchange of Ala107 by glutamate resulted in a strong decrease in the amount of recombinant protein produced in the expression host (data not shown). A purification of the mutant enzyme was impossible and a characterization failed. Since other mutations did not show a negative effect on the production of recombinant protein, a rapid degradation of the SMUL_1544 A107E variant was assumed. Whether the amino acid exchange hindered correct folding and caused a conformational change that induced proteolytic degradation can only be speculated. Interestingly, the replacement of the unique Ser242 with the conserved threonine resulted in an elevated activity with L-Thr-P and a decreased conversion rate with L-Ser-P (Tab. 3). The substrate preference of SMUL_1544 was not completely reversed, but the S242T variant displayed similar activities with L-Ser-P and L-Thr-P. From this result it can be concluded, that although the substrate preference of SMUL_1544 was not completely reversed, the Ser242 is definitely important for the unique substrate specificity of SMUL_1544.

Tab. 2: *In vitro* decarboxylation rates of SMUL_1544 and variant S242T.

	Specific activity (nkat/mg)	
Enzyme	L-Ser-P	L-Thr-P
Wild type	3.4 ± 0.7	0.7 ± 0.4
S242T	2.0 ± 0.4	2.6 ± 0.6

This finding raised the question, whether the exchange of Thr215 in *Se*CobD by a serine had a positive effect on the L-Ser-P conversion rate of the L-Thr-P decarboxylase. However, this was not the case, since the T215S variant of *Se*CobD displayed very low activities for both, L-Ser-P and L-Thr-P (<0.2 nkat/mg). Spectrophotometrical analysis of purified *Se*CobD T215S uncovered that less than 25 % of the protein molecules contained a PLP-cofactor. This finding indicated an improper binding of the cofactor to the mutant protein.

Does SMUL_1544 has decarboxylase and aminotransferase activity?

In the tests described above, the protein SMUL_1544 was identified as an L-Ser-P decarboxylase. Although the protein fulfilled the function of a CobD homolog in the norpseudo-B_{12} biosynthesis in *S. multivorans*, SMUL_1544 showed a higher amino acid identity to the putative aminotransferases of *Campylobacter jejuni* (46.2%, WP_052797156.1), *Pedobacter steynii* (46.6%, WP_074605868.1), or *Arcobacter butzleri* L354 (47.4%, KLE11288.1) than to the shorter *Se*CobD (22.4%, NP_459636.1). To better understand the phylogenetic affiliation of SMUL_1544 among the aspartate aminotransferase family of PLP-dependent enzymes, a phylogenetic analysis including various CobD and HisC homologs was performed (Fig. 3). As a third group of enzymes bacterial aspartate aminotransferases (AAT, EC 2.6.1.1) were included that perform the same aminotransferase reaction like HisC with aspartate instead of L-His-P as substrate (Kirsch *et al.* 1984, McPhalen *et al.* 1992). They formed a singly branch besides another one containing the CobD and HisC homologs. SMUL_1544 clustered with the putative aminotransferases of *Campylobacter jejuni*, *Pedobacter steynii*, and *Arcobacter butzleri* L354 (Smith and Muldoon 1975, Vandamme *et al.* 1992, Muurholm *et al.* 2007). The characterized HisC homolog of *E.coli* K-12 (CQR81539.1, Grisolia *et al.* 1985, Haruyama *et al.* 2001) as well as the putative HisC proteins of *S. enterica* (GAR68067.1) or *S. multivorans* (AHJ11577.1) are phylogenetically closer to SMUL_1544 than the group of CobD decarboxylases. Nevertheless a broad variability and partially low sequence identity among the CobD and HisC enzymes is seen in the sequence identity matrix (Tab. S1). The CobD homologs excluding SMUL_1544 showed a sequence identity of 30-80 %, but less than 30 % to the HisC proteins. All CobD and HisC homologs have less than 25 % identity to SMUL_1544. All tested aspartate aminotransferases showed less than 21 % sequence identity to the HisC and CobD homologs and thus occupied an evolutionary more distant position.

From the similarity of SMUL_1544 with selected putative aminotransferases one could deduce the presence of an aminotransferase activity of L-His-P in the enzyme besides its decarboxylation function. To test this hypothesis, the purified enzyme was analyzed with an HPLC-based aminotransferase assay. When L-His-P and the second substrate of HisC enzymes, 2-oxoglutarate, were applied as substrates for SMUL_1544 no formation of L-glutamate was observed, even after 24 h incubation time (Fig. S4). As a positive control for the aminotransferase assay HisC of *E.coli* K-12 was heterologously produced and purified. After incubation of enriched HisC with L-His-P and 2-oxoglutarate for 24 h at 37°C, most of the substrate was consumed and almost exclusively L-glutamate was detected (Fig. S4). This result proofed the assay functional. In conclusion SMUL_1544 does not possess L-His-P aminotransferase activity.

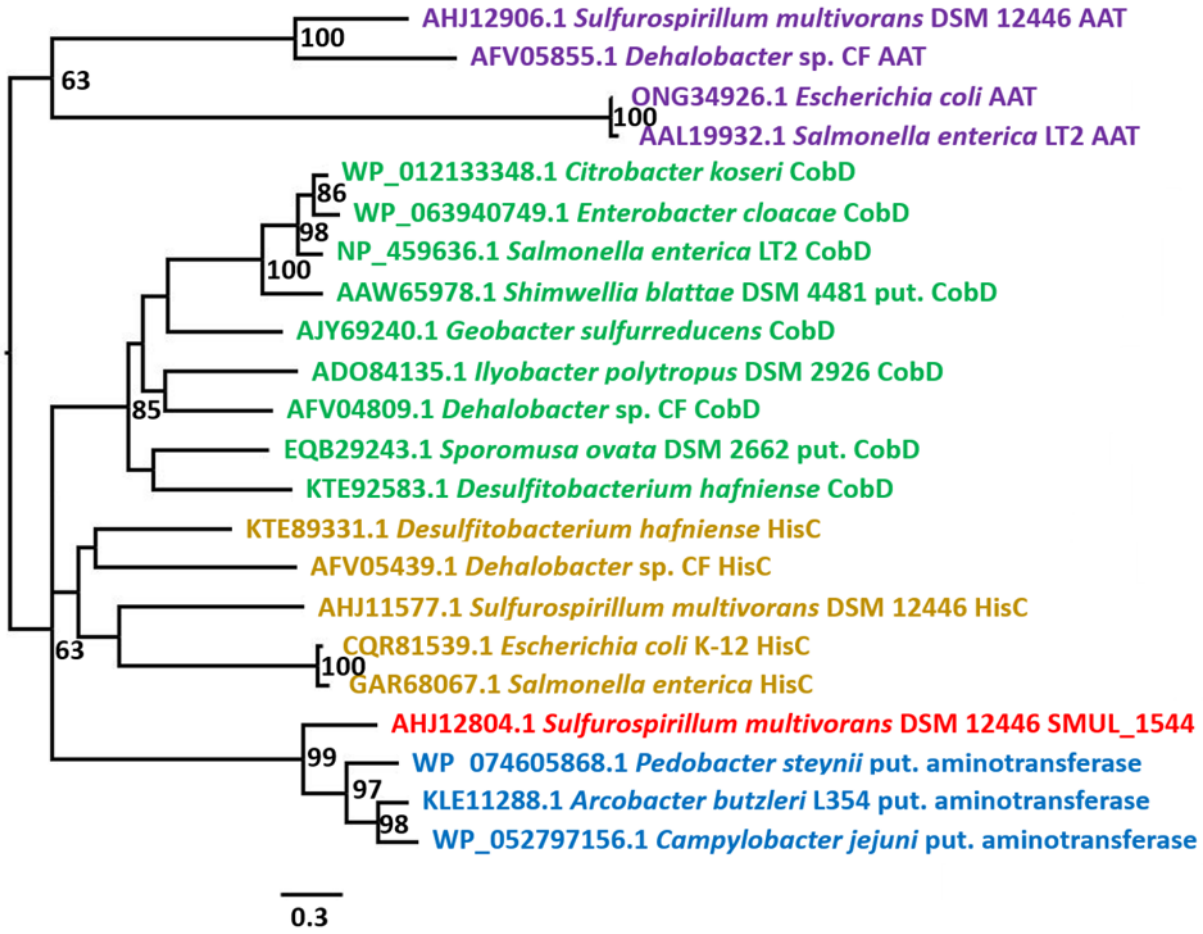

Fig. 3: Phylogenetic analysis of different CobD (*green*), HisC (*orange*), aspartate aminotransferases (AAT, *purple*), putative aminotransferases (*blue*), and SMUL_1544 (*red*).

Discussion

Cobamide cofactors consist of a conserved corrin ring and variable upper and lower ligands of the cobalt ion dependent on the respective enzyme (Kratky and Kräutler 1999, Randaccio *et al*. 2006, Gruber *et al.* 2011). The lower ligand is covalently connected to the corrin ring via a nucleotide loop containing a highly conserved(*R*)-1-aminopropan-2-ol *O*-2-phosphate (AP-P) in almost all biosynthesized cobamides (Warren *et al.* 2002). Only norpseudo-B_{12}, produced by *S. multivorans*, harbors an ethanolamine *O*-phosphate (EA-P) linker lacking a methyl group at position C176 of the cobamide (Kräutler *et al.* 2003). The biosynthesis of AP-P and its attachment to the corrin ring was first studied in *S. enterica* (Brushaber *et al.* 1998, Zayas *et al.* 2007, Fan and Bobik 2008). The L-threonine kinase PduX and the L-threonine *O*-3-phosphate (L-Thr-P) decarboxylase CobD were shown to convert L-threonine to AP-P, which is attached to the corrin ring by the adenosylcobinamide phosphate synthase CbiB. However, less was known about the EA-P biosynthesis in *S. multivorans*, which lacks a *pduX* gene in its genome (Goris *et al.* 2014). A previous study demonstrated the predicted CobD homolog SMUL_1544 as decarboxylase crucial for the EA-P fomation, since norpseudo-B_{12} was synthesized in an *S. enterica* Δ*cobD* strain containing heterologously expressed SMUL_1544 (Keller *et al.* 2016). In this study purified SMUL_1544 was finally identified as a novel EA-P forming L-serine *O*-phosphate (L-Ser-P) decarboxylase and thus as the CobD homolog of *S. multivorans*, henceforth named *Sm*CobD (Fig. 4). Interestingly, *Sm*CobD also decarboxylated L-Thr-P to AP-P with a fivefold lower activity than L-Ser-P *in vitro*. *In vivo* this AP-P linker synthesis became predominant when L-Thr-P was exogenously added to the medium at a high concentration of 1 mM (Keller *et al.* 2016). These results confirm that the cobinamide phosphate synthase CbiB of *S. multivorans* can incorporate EA-P as well as AP-P into cobinamide phosphate. This substrate variability is not surprising since the *S. enterica* CbiB homolog was already shown to have the same feature (Zayas *et al.* 2007). The origin of L-Ser-P as precursor of the EA-P linker in *S. multivorans* remains unclear. It might be formed from L-serine by an unknown kinase since a *pduX* gene, whose product synthesizes L-Thr-P in cobamide producers, is missing in *S. multivorans*. Alternatively L-Ser-P could be derived directly from the serine biosynthesis, where it serves as L-serine precursor (Pizer 1963). In this pathway 3-phospho-D-glycerate is converted to L-Ser-P by the activity of the two enzymes SerA (EC 1.1.1.95) and SerC (EC 2.6.1.52). L-serine is gained from L-Ser-P by the phosphatase

SerB (EC 3.1.3.3). In a proteomic study of *S. multivorans* grown on pyruvate and PCE the expression of *serA* and *serC* were found to be at least ten times higher compared to that of s*erB* (data not shown). These results indicate an enrichment of L-Ser-P and a low consumption to L-serine in the bacterium. Possibly this L-Ser-P pool serves as origin for the EA-P formation by *Sm*CobD and no specific L-serine kinase is required for cobamide biosynthesis in *S. multivorans*.

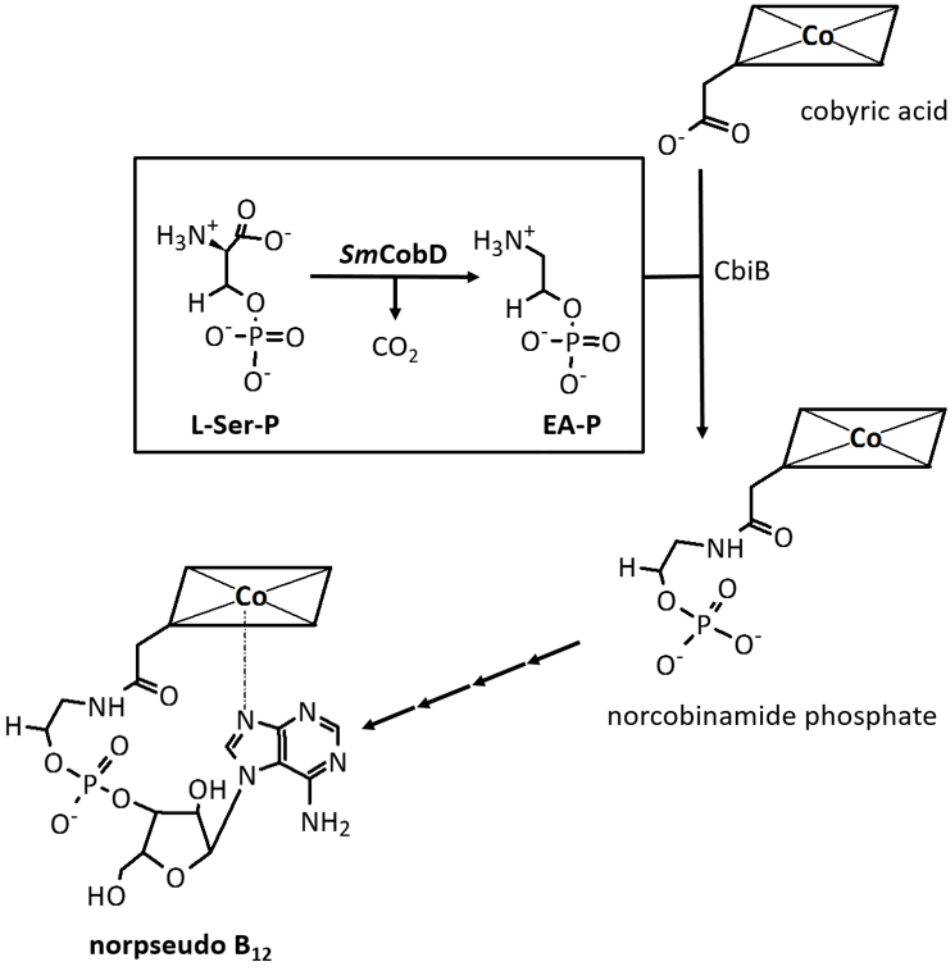

Fig.4: Nucleotide loop biosynthesis of norpseudo-B_{12} in *S. multivorans* with high-lighted linker formation by the L-Ser-P decarboxylase *Sm*CobD.

Interestingly, this study revealed that not only *Sm*CobD, but also *S. enterica* CobD (*Se*CobD), are able to convert L-Ser-P to EA-P and L-Thr-P to AP-P, respectively. Most probably this is a common feature for CobD enzymes among B_{12}-biosynthesizing prokaryotes. However, *Sm*CobD revealed a significant higher decarboxylation rate with L-Ser-P and *Se*CobD with L-Thr-P. Most CobD homologs are assumed to have a similar higher affinity for L-Thr-P over L-

Ser-P, since most bacteria and archaea produce AP-P-containing cobamides and not EA-P-norcobamides like *S. multivorans*. A previous study with *S. enterica* Δ*pduX*, grown under conditions that require the biosynthesis of cobamides for growth, revealed a low, but obvious growth of the bacterium (Fan and Bobik 2008). The authors suggested a second L-threonine kinase with a low affinity complementing PduX for some extent. With the newly gained knowledge that CobD homologues decarboxylate both, L-Ser-P and L-Thr-P, an utilization of L-Ser-P instead of L-Thr-P in case of Δ*pduX* might explain this low growth rate. This hypothesis could be proven by the isolation and identification of the cobamides produced by *S. enterica* Δ*pduX*, which would be norcobamides in case of EA-P formation by *Se*CobD.

The CobD proteins are pyridoxal-5'-phosphate (PLP) dependent decarboxylases and the active site of *Se*CobD was previously shown to bind PLP and L-Thr-P by several hydrogen bridge bonds mainly to the phosphate groups of both compounds (Cheong *et al.* 2002 a, b). By sequence comparison to *Se*CobD, most of these amino acids were also detected in *Sm*CobD except the PLP-phosphate binding Glu85 and Thr215 that are replaced by Ala107 and Ser242 in the *S. multivorans* enzyme, respectively. A *Sm*CobD variant A107E was unlike *Sm*CobD not heterologously expressed in *E. coli* BL21, indicating conformational changes resulting in a rapid degradation of the enzyme. When the unique Ser242 in *Sm*CobD was replaced by a threonine, which is highly conserved among L-Thr-P decarboxylases (Interpro: IPR005860, Finn *et al.* 2017), the L-Ser-P decarboxylation rate decreased and the L-Thr-P conversion rate increased resulting in an enzyme with similar activities towards both substrates. Hence, the amino acid Ser242 of *Sm*CobD was found to be crucial for the L-Ser-P conversion, but not the only determinant for the L-Ser-P preference. However, a replacement of Thr215 with a serine in *Se*CobD resulted in a low expression and almost non functional protein due to a low saturation with PLP. The binding site for PLP is somehow altered in case of *Se*CobD T215S hampering the correct incorporation of the cofactor. The N-terminus of *Sm*CobD is extended compared to *Se*CobD that is 28 amino acids shorter. The first 20 N-terminal amino acids of *Sm*CobD were shown to be non-essential for the activity of *Sm*CobD, since a deletion of these residues resulted in a 50 % reduced L-Ser-P decarboxylation rate. A subordinate role of the N-terminus for the correct conformation and the active site architecture is concluded. One highly important amino acid was confirmed with His26. A replacement of this histidine by alanine resulted in an almost non-functional protein. Since the H26A variant was completely saturated

with PLP an inability in incorporating the substrates L-Ser-P or L-Thr-P might cause these low activity. Thus, His26 of *Sm*CobD is assumed to be the analog of His8 of *Se*CobD, whose importance for the binding of L-Thr-P was affirmed in the three-dimensional structure of the protein (Cheong *et al.* 2002 a, b). In the applied phylogenetic study *Sm*CobD showed a higher similarity to putative, but uncharacterized aminotransferases of *Pedobacter steynii*, *Campylobacter jejuni* and *Arcobacter butzleri* L354 than to other CobD decarboxylases (Fig. 3). These three enzymes contain a serine residue at the position of Ser242 and most active site residues specific for CobD homologs. Probably they are L-Ser-P decarboxylases and not aminotransferases. *Campylobacter jejuni*, *Pedobacter steynii* and *Arcobacter butzleri* L354 are not known to synthesize cobamides *de novo* due to the lack of most cobamide biosynthesis genes in their genomes. Thus, the presence of a putative L-serine *O*-phosphate decarboxylase producing ethanolamine *O*-phosphate in these organisms might be connected to the glycerophospholipid metabolism (Martínes-Morales *et al.* 2003). Phylogenetically *Sm*CobD displayed higher similarities to HisC aminotransferases than to other CobD homologs. However, the *S. multivorans* enzyme showed no aminotransferase activity for L-histidinol phosphate. Probably *SmcobD* is evolutionary derived from certain *hisC* genes, whose mutations caused the protein to loose the aminotransferase and gain a decarboxylation function. Maybe it is a link between HisC and CobD enzymes, but this is difficult to predict, due to the low sequence identities of *Sm*CobD to these proteins. This study unraveled the EA-P linker biosynthesis pathway of *S. multivorans*, which is an analog of the AP-P pathway of *S. enterica*. The L-Ser-P decarboxylase *Sm*CobD was identified as key player in this process. The structural analysis of this enzyme with L-Ser-P or L-Thr-P in the active site was not successful so far, but is currently under investigation. When available these data will reveal why *Sm*CobD prefers L-Ser-P over L-Thr-P.

Experimental procedures

Chemicals and bacteria

All chemicals used in this study, if not otherwise stated, were purchased from Sigma Aldrich, Germany in the highest available purity. The *E. coli* strains Dh5α and BL21 (DE3) were obtained from the DSMZ (German collection of microorganisms and cell cultures).

Genetic approaches

The *SMUL_1544* gene was cloned into the vector pASK-IBA-63c-plus (IBA GmbH, Goettingen, Germany) from genomic DNA of *Sulfurospirillum multivorans* by use of the primers F1 and R1 (Tab. 3). The resulting plasmid p1544 was transformed into *E. coli Dh5α* via the heat-shock method (Froger and Hall 2007). After sequence verification (Macrogen, Amsterdam, The Netherlands) p1544 was transformed into *E. coli* BL21 (DE3). Simultaneously N-terminal truncations of SMUL_1544 lacking the nucleotides for the amino acid 2-6, 2-11, 2-16, 2-21, and 2-29, respectively, the point mutations H26A, A107E, S242T, the *cobD* gene of *S. enterica* (plasmid pCobD) and its variant T215S were cloned in the same way. For the point mutations of SMUL_1544 and *Se*CobD the plasmid p1544 or pCobD served as template and three PCR were applied. (I) and (II) created two fragments (upstream and downstream part of the gene) with a complementary region that contained the respective mutation.

Tab. 3: Forward (F) and reverse (R) primers used for cloning. The corresponding R-primers for the site-directed mutagenesis primers are reverse complement to F9-12. The mutation sites are underlined.

Nr	Sequence (5'-3')	Product
	flanking primer	
F1	AGCGTCATGATTGATACAATGAATGCACG	SMUL_1544
F2	AGTTCATGACACGTAACACTCAATTTACG	Δaa 2-6
F3	GCTATCATGATTACGAAAGCGTTTCATGC	Δaa 2-11
F4	AGCTTCATGAATGCGTTAAAACAGAATGC	Δaa 2-16
F5	TCAGTCATGAATGCGGGAAGTCATAGTCC	Δaa 2-21
F6	ATGCTCATGACTTGAAAAAAATGTTCC	Δaa 2-29
F7	AGCTCCATGGCGTTATTCAACAGCGCGCATGG	*Se* CobD
F8	AGCTTCATGAGCACGGTGACTATTACCGATTTAGC	*Ec* HisC
R1	AGCGCTCGAGCTTACTATCCTTTAGTATG	SMUL_1544
R2	ACTGCTCGAGATCAGCAGGGGCTATACCGG	*Se* CobD
R3	TGCACTCGAGAACTTGCTCCGCACGTAAGGCG	*Ec* HisC
	site-directed mutagenesis primer	
F9	GAATGCGGGAAGTGCAAGTCCAAGTATGG	*Sm* _H26A
F10	GAATGGTGAGACTGAAATTATTCAG	*Sm* _A107E
F11	TTAAAAGTCTCACTAAAGATTTTGG	*Sm* _S242T
F12	TTCGCTGAGCAAATTTTATGCCATTCC	*Se* _T215S

The primers used for this approach were each a flanking primer (start or end of gene) and a primer containing the mutation (Tab. 3). Both primers harboring the mutation (F9-12) are

reverse complements of each other (The reverse complemented R-primers are not shown in Tab.3). The third PCR combined both fragments with help of the flanking primers of the gene added after 10 cycles without primers. *SecobD* was cloned from genomic DNA of *S. enterica*. The gene encoding HisC from *E.coli* K-12 was cloned into pASK-IBA63c-plus from genomic DNA of the bacterium.

Purification of the recombinant proteins

E. coli BL21 (DE 3) containing the *SecobD*, *SMUL_1544* or *EchisC* plasmids was cultivated in LB medium (Bertani *et al*. 1951) supplemented with 100 µg/ml ampicillin (2 % inoculum). The cultivation was performed at 37 °C and 100 rpm shaking under aerobic conditions. After the bacteria reached an OD at 550 nm of 0.5, protein expression was induced by addition of 200 ng/ml anhydrotetracycline. After three further hours incubation at 37 °C the cells were harvested (8000 rpm, 10 min, 10 °C) and the pellets stored at -20 °C. For cell disruption the cells were resuspended in 1 ml/g cells of 50 mM Tris-HCl pH 7.9 containing protease inhibitor (Roche, Mannheim, Germany). The resuspension was then incubated at 37 °C, 30 min, and 500 rpm in the presence of 10 mg/ml lysozyme and DNase I. Subsequently the lysozyme-treated cells were shock frozen in liquid nitrogen and then thawed at 37 °C, 1000 rpm for 15 minutes. The crude extract was gained by centrifugation (1 h, 13000 rpm, 4 °C). The recombinant proteins were purified by Streptactin affinity chromatography with 10 ml of washing buffer (100 mM Tris-HCl pH 8.0) and 5 ml elution buffer (100 mM Tris-HCl pH 8.0, 2.5 mM desthiobiotin) according to the manufacturer's recommendations. The eluted proteins were concentrated and the Tris buffer was replaced with 50 mM HEPES pH 7.9.

***In vitro* decarboxylation or aminotransferase assay**

The CobD decarboxylation assay was performed modified from the method described by Brushaber *et al.* 1998. In this assay 2000 nmol L-Ser-P or L-Thr-P and 50 nmol PLP were mixed in 500 µl 50 mM HEPES pH 7.9. The reaction was started by addition of 50 µg of a protein (e.g. SMUL_1544, *Se*CobD). The L-His-P aminotransferase assay contained 2000 nmol L-His-P, 2000 nmol 2-oxoglutarate, 50 nmol PLP in 500 µl 50 mM HEPES pH 7.9. In all cases a sample without

enzyme was simultaneously prepared. The samples were incubated at 37 °C (if not stated otherwise for 1 h) and the reaction was then stopped by placing the tubes on 100 °C for 5 min. After cooling down on ice, the samples were centrifuged (5 min, 13200 rpm) and 450 µl supernatant was applied to HPLC analysis (Knauer Smartline systems). For derivatization the samples were mixed with 100 µl 0.5 M potassium borate and 50 µl derivatization solution (70 µM o-phthaldialdehyde (oPA), 140 µM mercaptoethanol, 22 M methanol, 5 mM potassium borate pH 9.2). After a two minutes incubation time at room temperature, 100 µl of the derivatized sample was transferred into 500 µl washing solution (50 mM sodium acetate pH 6.1, 20 % methanol). Finally 50 µl of this mix was applied to HPLC. The stationary phase was a Chromolith ® performance RP-18e, 100-4.6 mm column (Merck) with a Chromolith ®, RP-18e, 5-4.6 mm guard column. The mobile phases were (A) tetrahydrofuran: methanol: 50 mM sodium acetate pH 5.9 (1:2:97) and (B) methanol: 50 mM sodium acetate pH 5.9 (8:2). The flow rate was 0.7 ml/min and the column temperature was set to 30 °C. The detector was a diode array detector and the absorption was recorded at 340 nm. The program was 5 min 100 % A, 15 min gradient to 75 % A, 5 min gradient to 0 % A, 5 min 0 % A, 5 min gradient to 100 % A, 10 min 100 % A, all together 45 min. The peaks were compared with L-Ser-P, L-Thr-P, EA-P, L-His-P and L-glutamate standards. The AP-P standard was gained through fractionation of the L-Thr-P decarboxylation product peak of *Se*CobD from the HPLC. The enzyme activities were calculated with the consumption of L-Ser-P or L-Thr-P. To further identify the enzyme products as EA-P or AP-P on HPLC the reaction mixtures containing SMUL_1544 with L-Ser-P or *Se*CobD with L-Thr-P were subjected for alkaline de-phosphorylation after precipitation of the enzymes. Thus 200 µl of the reaction mixtures were mixed with 47 µl buffer (100 mM CHES pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$ x 6 H_2O, 1 % Tween 20) and 3 µl alkaline phosphatase. After 1 h incubation at 37 °C, the reaction was stopped (5 min, 100 °C) and after centrifugation 200 µl were applied to HPLC (Fig. S2-S3). The signals were compared with L-serine, L-threonine, ethanolamine and 1-aminopropan-2-ol standards.

UV/Vis-spectroscopy of SMUL_1544 (wildtype, H26A) and *Se*CobD_T215S

To determine the saturation of the PLP-dependent enzymes with PLP, the proteins were mixed in 100 µl 50 mM HEPES pH 7.9 to a concentration of 11 µM monomer (dimer concentration 5.5 µM) each. By addition of 100 µM PLP and a 5 min incubation time at room temperature

the enzyme solutions were saturated with cofactor. With help of several dilution steps in 10 kDa concentrator vials by use of 50 mM HEPES pH 7.9 the calculated concentration of free PLP was reduced to less than 0.4 µM. By UV/Vis-spectroscopy the wavelengths of 388nm (PLP) and 280 nm (protein) were determined in a quartz cuvette (d = 1 cm). With help of the molar extinction coefficient of PLP ($\varepsilon_{388\ nm}$ = 4.9 mM^{-1} cm^{-1}) the concentration of PLP in the enzyme fractions was determined. The factor A(280 nm)/A(388 nm) was determined and compared to the value obtained for SMUL_1544 (5.4).

Alignments and phylogenetic tree

For the phylogenetic tree the protein sequences of different annotated bacterial HisC and CobD proteins as well as aspartate aminotransferases were downloaded from NCBI. The alignment and phylogenetic tree was created by use of MEGA7 (Kumar, Stecher, and Tamura 2015). First a MUSCLE alignment of the protein sequences was performed. Afterwards the phylogenetic tree was created with the Maximium likelihood method and the bootstraps method with 1000 replications. The format refinement of the phylogenetic tree was done with FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software /figtree/). For the pairwise alignment of SMUL_1544 and *Se*CobD MUSCLE (v3.8) was used (Edgar 2004a, Edgar 2004b).

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Functional analysis of a unique L-serine *O*-phosphat*e* decarboxylase essential for the norcobamide biosynthesis in *Sulfurospirillum multivorans*

Supporting Information

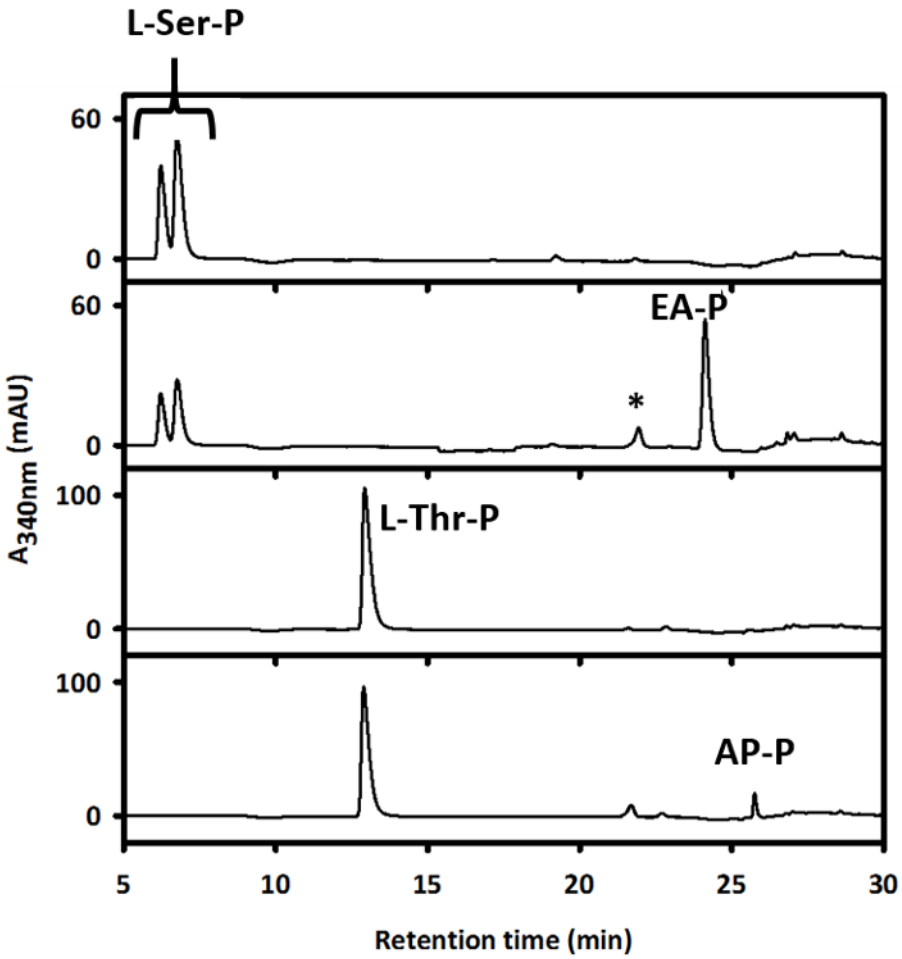


Fig. S1: HPLC analysis of the SMUL_1544 *in vitro* decarboxylation products after 1 h incubation at 37 °C. The reaction mixtures contained either L-Ser-P without (*first trace*) or with SMUL_1544 (*second trace*), or L-Thr-P without (*third trace*) or with SMUL_1544 (*last trace*). The star (*) represents remaining fractions of Tris in the SMUL_1544 sample.

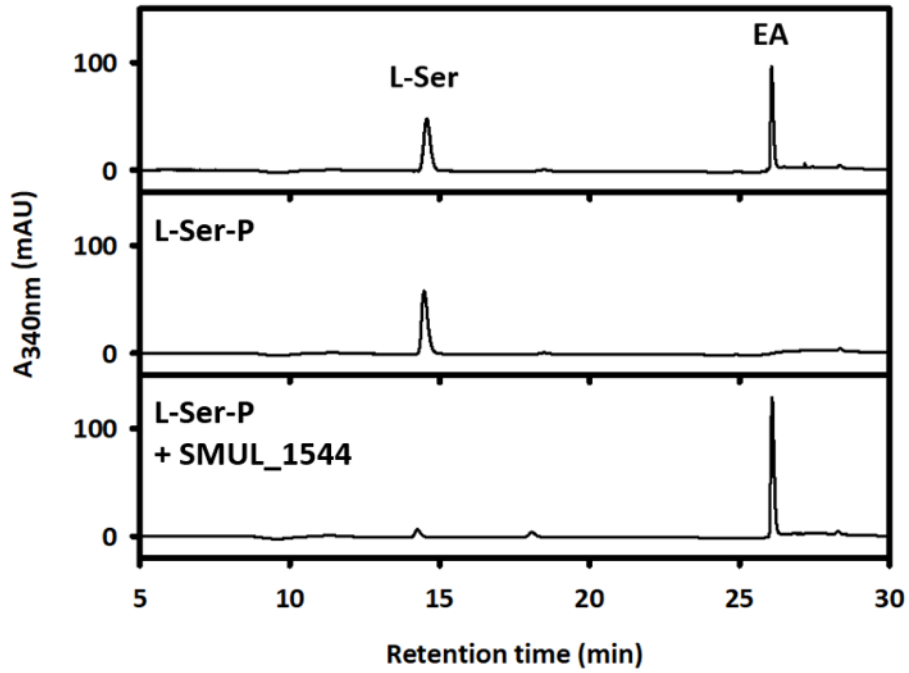


Fig. S2: HPLC elution profiles of the reaction products with L-Ser-P as substrate in the absence (*second trace*) or presence of SMUL_1544 (*third trace*) after treatment with alkaline phosphatase. L-Ser, L-serine; Ea, ethanolamine (2-aminoethanol).

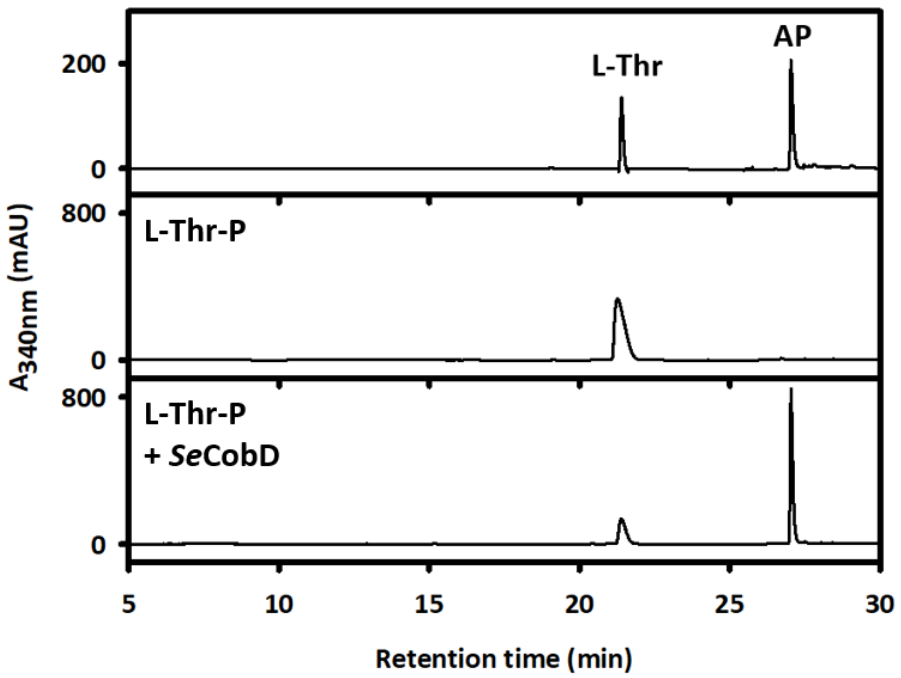


Fig. S3: HPLC elution profiles of the reaction products with L-Thr-P as substrate in the absence (*second trace*) or presence of *Se*CobD (*third trace*) after treatment with alkaline phosphatase. L-Thr, L-threonine; AP, 1-aminopropan-2-ol.

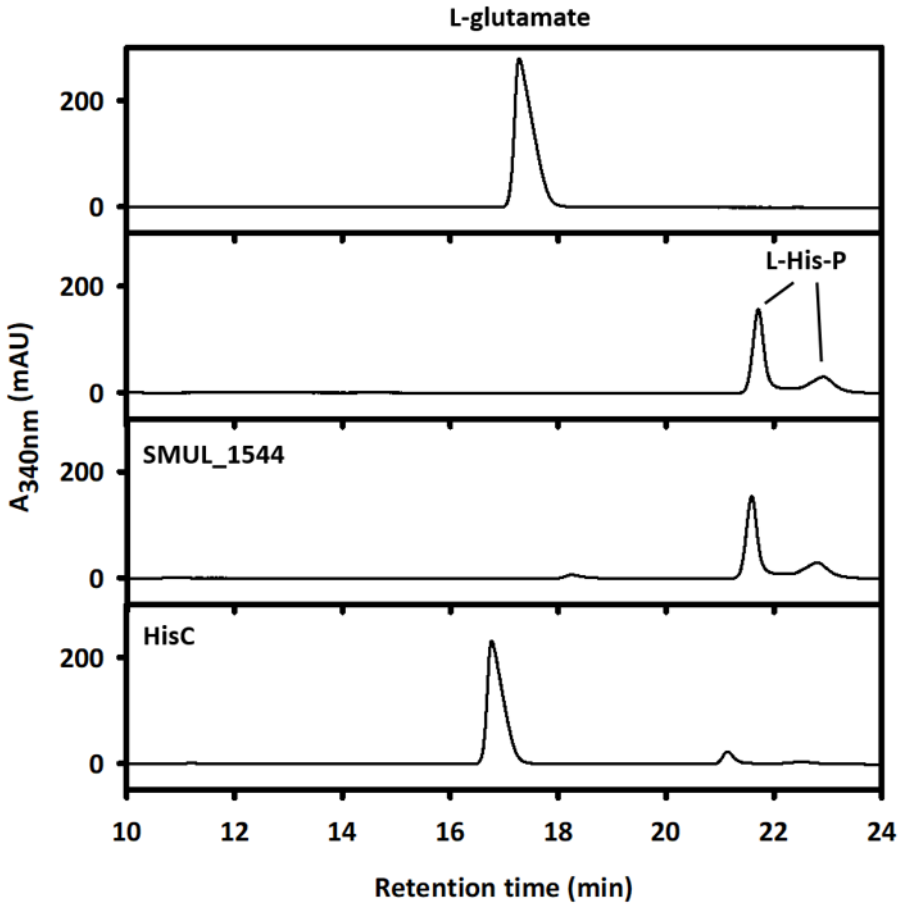


Fig S4: HPLC elution profiles of aminotransferase reactions with L-His-P and 2-oxoglutarate in the presence of SMUL_1544 (*third trace*) or HisC (*E. coli* K-12, *last trace*) after 24 h at 37 °C.

Tab. S1: Sequence identity matrix of the multiple sequence alignment for phylogenetic analysis (Fig. 3). The numbers represent percent amino acid identity. Sequences 1-4 belong to aspartate aminotransferases (AAT), 9-13 to L-histidinol phosphate aminotransferases (HisC) and 14-22 to L-threonine *O*-3-phosphate decarboxylases (CobD). The *yellow line* (sequence 5) belongs to SMUL_1544 (*Sm*CobD) and the *blue line* (sequence 20) to *Se*CobD. The *green lines* belong to the putative aminotransferases (put. AT) from *Pedobacter steynii* (sequence 6), *Arcobacter butzleri* L354 (sequence 7) and *Campylobacter jejuni* (sequence 8).

		AAT				SMUL_1544 + put. AT				HisC					CobD									
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	ONG34926.1	100	96	15	16	16	18	17	15	17	16	16	15	15	15	15	16	15	17	15	17	17	16
2	AAL19932.1	96	100	15	17	16	19	17	15	18	16	16	15	15	15	16	16	14	17	15	16	17	15
3	AHJ12906.1	15	15	100	36	19	16	17	16	16	18	19	17	20	19	17	16	17	19	17	20	19	19
4	AFV05855.1	16	17	36	100	15	17	14	14	19	19	19	18	15	18	15	16	15	17	16	15	15	17
5	AHJ12804.1	16	16	19	15	100	47	47	46	21	25	24	21	26	25	23	23	21	24	22	22	23	24
6	WP_074605868.1	18	19	16	17	47	100	57	56	22	23	23	21	27	23	21	23	24	28	21	23	21	23
7	KLE11288.1	17	17	17	14	47	57	100	68	20	21	21	21	26	24	23	22	21	27	18	22	22	22
8	WP_052797156.1	15	15	16	14	46	56	68	100	21	22	22	21	25	24	22	21	22	26	20	21	23	21
9	AFV05439.1	17	18	16	19	21	22	20	21	100	27	27	25	26	25	26	24	26	23	22	22	23	23
10	CQR81539.1	16	16	18	19	25	23	21	22	27	100	90	25	29	25	23	23	29	24	24	23	23	23
11	GAR68067.1	16	16	19	19	24	23	21	22	27	90	100	24	29	25	21	23	27	24	24	24	23	23
12	AHJ11577.1	15	15	17	18	21	21	21	21	25	25	24	100	29	21	25	21	23	26	21	22	20	19
13	KTE89331.1	15	15	20	15	26	27	26	25	26	29	29	29	100	29	27	28	31	29	29	28	29	27
14	AFV04809.1	15	15	19	18	25	23	24	24	25	25	25	21	29	100	33	37	37	33	37	35	34	35
15	KTE92583.1	15	16	17	15	23	21	23	22	26	23	21	25	27	33	100	41	36	36	31	35	33	33
16	EQB29243.1	16	16	16	16	23	23	22	21	24	23	23	21	28	37	41	100	40	36	37	36	34	36
17	AJY69240.1	15	14	17	15	21	24	21	22	26	29	27	23	31	37	36	40	100	36	41	40	38	39
18	ADO84135.1	17	17	19	17	24	28	27	26	23	24	24	26	29	33	36	36	36	100	41	38	36	36
19	AAW65978.1	15	15	17	16	22	21	18	20	22	24	24	21	29	37	31	37	41	41	100	60	61	60
20	NP_459636.1	17	16	20	15	22	23	22	21	22	23	24	22	28	35	35	36	40	38	60	100	77	74
21	WP_012133348.1	17	17	19	15	23	21	22	23	23	23	23	20	29	34	33	34	38	36	61	77	100	81
22	WP_063940749.1	16	15	19	17	24	23	22	21	23	23	23	19	27	35	33	36	39	36	60	74	81	100

3 Discussion

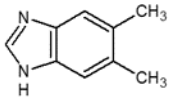
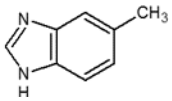
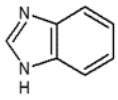
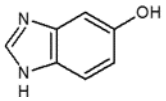
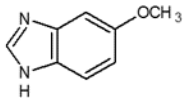
The Gram-negative ε-proteobacterium *Sulfurospirillum multivorans* belongs to the phylogenetically diverse group of organohalide respiring bacteria (OHRB) that couple the reductive dehalogenation of organohalogens to energy conservation (Neumann *et al.* 1994, Fetzner 1998, Smidt and de Vos 2004, Hug *et al*. 2013). This organism reduces the abundant environmental pollutant tetrachloroethene to *cis*-1,2-dichloroethene in two subsequent dechlorination steps, a reaction catalyzed by the tetrachloroethene reductive dehalogenase PceA (Neumann *et al.* 1994, Scholz-Muramatsu *et al.* 1995, Bommer *et al.* 2014). This iron-sulfur protein harbors the unusual cobamide cofactor norpseudo-B_{12}, which bears an adenine moiety as lower base and a unique ethanolamine-phosphate (EA-P) linker in its nucleotide loop (Kräutler *et al.* 2003; Fig. 1.3). The PceA homolog of *S. multivorans* displayed a tremendously higher PCE and TCE dechlorination activities than other PceA enzymes from bacteria that use cobamide cofactors with an (*R*)-1-aminopropan-2-ol O-2-phosphate (AP-P) linker and a lower ligand different from adenine (Schubert and Diekert 2016). Norpseudo-B_{12} was shown to be buried deep inside the PceA enzyme in a special 'base-off' conformation, in which the adenine and the ribose moiety of the nucleotide loop are curled and undergo hydrogen bonding with the corrin ring and the EA-P linker besides the protein backbone (Bommer *et al.* 2014). The cobamide cofactor is biosynthesized *de novo* in a more than 25 enzymes involving pathway by *S. multivorans* and most of the responsible genes are clustered in one consecutive operon in the genome of the bacterium (Goris *et al.* 2014). The question was, if norpseudo-B_{12} with its specific structure was essential to the function of PceA in *S. multivorans* and how the *de novo* cobamide biosynthesis in this organism differed from that of other bacteria.

Guided biosynthesis of norcobamides with exogenous benzimidazoles in *S. multivorans*

The amendment of *S. multivorans* with increasing concentrations of the benzimidazole derivatives DMB, Bza, 5-MeBza, 5-OHBza, or 5-OMeBza led to an increasing production of the benzimidazole-containing norcobamides (NCba) with a coherent decrease in the norpseudo-B_{12} synthesis (2.2, Fig. 1; Tab. 3.1). At exogenous concentrations of 25 µM all tested benzimidazoles completely replaced the adenine moiety of norpseudo-B_{12} in *S. multivorans*

except 5-OMeBza. In this case still 10 % adenine-containing norcobamide was left. This ability of certain bacteria to synthesize cobamides with a variety of benzimidazole or purine ligands was already shown for the OHRB *Desulfitobacterium hafniense* Y51 and for other bacteria like *Salmonella enterica* and *Shimwellia blattae* (Crofts *et al.* 2013, Schubert T. unpublished data). Contrary to these findings other bacteria displayed a restricted incorporation of lower ligand bases during cobamide biosynthesis indicating a selectivity of the cobamide-dependent enzymes for certain lower bases. From exogenous adenine and benzimidazole derivatives exclusively adenine was incorporated into a cobamide by the firmicute *Lactobacillus reuteri* and only the benzimidazoles by *D. mccarty*i, *Sinorhizobium meliloti*, and *Veillonella parvula* (Crofts *et al.* 2013). The latter has two lower ligand activating phosphoribosyltransferases, the homodimeric CobT and the heterodimeric ArsAB (Gronow *et al.* 2010, Crofts *et al.* 2013). With ArsAB it is able to synthesize cobamides with phenolics (i.e. phenol, p-cresol) as lower ligands, a feature it shares with the better studied bacterium *Sporomusa ovata* (Stupperich *et al.* 1989, Stupperich and Eisinger 1989, Chan and Escalante-Semerena 2011). Since *S. multivorans* has a CobT and not an ArsAB homolog its inability in synthesizing p-cresolyl norcobamide with exogenous p-cresol is not surprising. The nicotinate mononucleotide:5,6-dimethylbenzimidazole phosphoribosyltransferase CobT of *S. multivorans* (*Sm*CobT) was able to activate all tested benzimidazoles (2.2, Fig. 3; Tab. 3.1) including 5,6-DOMeBza and 5,6-DNO_2Bza as well as adenine into their specific α-ribotides *in vitro* (Fig. 1.2). The highest activation rates were detected for adenine and 5-OHBza and five times lower ones for the methylated benzimidazoles DMB and 5-MeBza (Tab. 3.1). The methyl group 5-OMeBza has in addition of 5-OHBza dropped the CobT activity 2.5 fold. Seemingly the enzyme works best with hydrophilic substrates, which is atypical compared to yet characterized CobT enzymes. CobT of *Clostridium sticklandii* activated Bza five times faster than adenine, although the K_M value for adenine was 50 times lower than for Bza (Fyve and Friedmann 1969). The active site of *Salmonella enterica* CobT (*Se*CobT) in complex with adenine, DMB, 5-OMeBza, or 5-MeBza revealed a highly hydrophobic lower ligand binding pocket including four leucine, a isoleucine, a proline, a valine and a methionine residue (Cheong *et al.* 1999, Cheong *et al.* 2001). Only two hydrophilic side chains displayed hydrogen bridge bonds to the hydrophilic adenine (Ser80 and Gln88) allowing it to bind at almost the same position like DMB in the active site (Cheong *et al.* 2001).

Table 3.1: Guided biosynthesis and effects of norcobamides (NCba) with exogenous benzimidazoles in *S. multivorans*. The CobT activation data are derived from the *in vitro* assay. The structure of the bases of the α-ribotide (RP) products of *Sm*CobT, the NCba as produced by the cell, and the NCba as isolated from purified PceA with the ratio in brackets is shown. The effects of 25 µM exogenous benzimidazoles on the PCE metabolism. *Green arrows* indicate no and *red arrow* indicate little and significant negative effects.

	DMB	**5-MeBza**	**Bza**	**5-OHBza**	**5-OMeBza**
Structure					
Spec. CobT activity (nkat/mg)	0.29 ± 0.04	0.23 ± 0.04	0.91 ± 0.06	1.34 ± 0.05	0.56 ± 0.05
'base' α-RP by CobT *in vitro*	DMB	5-MeBza (2) 6-MeBza (1)	Bza	5-OHBza (1) 6-OHBza (9)	5-OMeBza (2) 6-OMeBza (1)
'base' NCba in cells (25 µM)	DMB	5-MeBza (2) 6-MeBza (1)	Bza	6-OHBza	5-OMeBza (9) adenine (1)
'base' NCba in PceA (25 µM)	DMB	5-MeBza (1) 6-MeBza (1)	Bza	6-OHBza	5-OMeBza (9) adenine (1)
% PceA occupied with NCba	1.5	100	100	100	100
Effect on PCE-growth	↓	→	→	→	→
Effect on PceA activity	↓	↓	→	→	→
Effect on PceA processing	↓	↓	→	→	→

The three dimensional structure of *Sm*CobT is not known yet, but a model by use of the i-tasser server with *Se*CobT as template was generated (Zhang 2008, Roy *et al.* 2010, Yang *et al.* 2015). The *S. multivorans* enzyme has residue Ser77 and Val85 at the same position like Ser80 and Gln88 of *Se*CobT. Thus, only one predicted hydrophilic connection to adenine is available. However the reason, why *Sm*CobT in comparison to *Se*CobT and the CobT homologs of several other bacteria works best with hydrophilic substrates hypothetically lies in the shorter C-terminus (Fyve and Friedmann 1969, Crofts *et al.* 2013, Hazra *et al.* 2013). *Sm*CobT lacks the highly hydrophobic C-terminus of *Se*CobT contributing to binding of the lower ligands in the active site (Fig. 3.1). A similar hydrophobic C-terminus is present in the CobT homologs of *C.*

sticklandii that already showed to prefer hydrophobic bases and in *D. mccartyi* VS and *D. hafniense* Y51. These enzymes might also convert hydrophobic benzimidazoles like DMB and 5-MeBza faster than 5-OHBza or purines.

```
Se        AMRLGEGSGAALAMPIVEAACAMFHNMGELAASNIVLPEGNANAT
Sm        NMKLGEGSGAVLVFPLVEAASNLTRDIRVYPEV------------
Vp        QMSMGEGTGAILMVQMLKTTQYMFVNVGTFADLMKL---------
Sim       GMRLGEGTGAALAAGIVKAAAACHSGMATFAQAGVSNKE------
Cs        DMRLGEGSGAALCFNIIEAANFTYKNMYTFEEAGFTV--------
Dm        DMRLGEGTGAALAMNIIEASNRIQHEMASFADAGVSEKQS-----
Dh        DFRLGEGTGAAIAFHLLDASIRILNEMATFESAGVSGKN------
            : :***:** :    ::.::       :
```

Fig. 3.1: Sequence alignment of the C-termini of the CobT homologs of *Salmonella enterica* ser. *Thyphimurium* LT2 (Se), *Sulfurospirillum multivorans* DSM 12446 (Sm), *Veillonella parvula* (Vp), *Sinorhizobium meliloti* (Sim), *Clostridium sticklandii* (Cs), *Dehalococcoccoides mccartyi* VS (Dm) and *Desulfitobacterium hafniense* Y51 (Dh). The conserved glutamate residues crucial for CobT activity are colored in *cyan* and the hydrophobic residues contributing to the binding of the lower ligand in *Se*CobT in *yellow*. GenBank accession numbers: Se, NP_460961.1; Sm, AHJ12807.1; Vp, EFB86114.1; Sim, AEH78791.1; Cs, CBH20412.1; Dm, ACZ61744.1 ;Dh, BAE83903.1. The sequence alignment was performed with Clustal Omega (Sievers *et al.* 2011, McWilliam *et al.* 2013, Li *et al.* 2015).

If the C-terminus of CobT enzymes is responsible for the substrate preference could by analyzed via mutagenesis. The singly substituted benzimidazoles 5-OHBza, 5-MeBza, and 5-OMeBza were activated to two different ribotides harboring the substituent either at C5 or C6 of the benzimidazole by *Sm*CobT *in vitro* (2.2, Fig. 4; Tab. 3.1). Interestingly two-thirds of 5-MeBza and 5-OMeBza were converted to the 5-(Bza) α-ribotide (α-RP) and 90 % of 5-OHBza to the 6-OHBza α-RP. This finding reveals a regioselectivity for the different substituents by CobT. In a previous study *Se*CobT revealed a similar predominantly formation of 5-OMeBza α-RP and 6-OHBza α-RP from 5-OMeBza and 5-OHBza, respectively (Crofts *et al.* 2014). However, CobT of *Veillonella parvula* converted 5-OHBza and 6-OMeBza in both cases predominantly to the 6-ribotide and the activation products of CobT from *Sinorhizobium meliloti* were mainly 5-ribotides. Actually it is difficult to assume, which residues inside the CobT enzymes are responsible for these different activation patterns of singly substituted benzimidazoles due to the lack of structures containing these benzimidazoles. Thus, the determination of the three dimensional structures of some CobT with 5-OHBza, 5-MeBza, and 5-OMeBza could gain us new insights in this actual topic of cobamide biosynthesis. During cobamide biosynthesis the

α-ribotides are combined with cobinamide-GDP by CobS and finally the 5'-phosphate group of the cobamide is removed by the cobalamin/α-ribazole phosphatase CobC yielding norpseudo-B_{12} in *S. multivorans* (Maggio-Hall *et al.* 2004, O'Toole *et al.* 2004, Zayas and Escalante-Semerena 2007). For a long time it was assumed that these enzymes perform their activities independent from the structure of the base of the α-ribotides (Trzebiatowski *et al.* 1997). However, in a previous study it was affirmed that although *Se*CobT synthesized both 5-OMeBza and 6-OMeBza α-RP from 5-OMeBza *in vitro*, only 5-OMeBza-Cba was produced by guided biosynthesis in *S. enterica* (Crofts *et al.* 2014). *Veillonella parvula*, whose CobT formed predominantly 6-OHBza α-RP, synthesized almost exclusively 5-OHBza-Cba from exogenous 5-OHBza. With this study *S. multivorans* was described as the first bacterium that exclusively produced 6-OHBza-NCba from both OHBza-ribotides and only 5-OMeBza-NCba from both OMeBza-ribotides. Moreover, the first evidence for the regioselectivity of a CobT towards MeBza was gained with the investigation of *Sm*CobT. Even more interesting is the phenomenon that both MeBza-ribotides are incorporated into norcobamides in *S. multivorans* in the same ratio as they were produced by CobT (Tab. 3.1). Thus, either CobS or CobC are unable to convert substrates containing 6-OMeBza or 5-OHBza, but non-selective in case of 5- or 6-MeBza. Another example for the selectivity for CobT products was unraveled in *L. reuteri* and *S. meliloti* that synthesized only adenine-Cba or several benzimidazole-Cba, respectively, although their CobT homologs efficiently activated adenine and benzimidazoles *in vitro* (Crofts *et al.* 2013, Hazra *et al.* 2013). Since CobC is a phosphatase removing the 5'-phosphate group from the α-ribotides or from the cobamide-5'-phosphate, likely the membrane protein CobS is selective for certain CobT products (O'Toole *et al.* 2004, Zayas and Escalante-Semerena 2007). So far every attempt to purify and analyze CobS proteins failed, but an in depth analysis of the *S. multivorans* CobS might unravel the predicted selectivity for certain α-ribotides. At 25 µM in the medium all tested benzimidazoles replaced the adenine moiety of norpseudo-B_{12} in *S. multivorans* almost completely except the methoxylated 5-OMeBza and 5,6-DOMeBza. While 5-OMeBza was incorporated into 90 % of the NCba molecules under these conditions, only 2 % 5,6-DOMeBza-NCba norseudo-B_{12} was produced in the bacterium with exogenous. However, 5,6-DOMeBza was efficiently activated into an α-ribotide by the *in vitro* CobT assay (Keller, data not shown). These findings are pointing towards an inefficient uptake of exogenous methoxylated benzimidazoles in *S. multivorans* compared to methylated or hydroxylated ones that replaced the adenine base of norpseudo-B_{12} more efficiently. However

a second fate of the methoxylated benzimidazoles in *S. multivorans* besides the cobamide biosynthesis cannot be excluded, but there is no other known biological function of benzimidazoles. Diffusion through the cell wall is assumed as uptake mechanism of exogenous benzimidazoles into bacteria. Proteomic data of *S. multivorans* amended with DMB revealed an uncharacterized phosphoribosyltransferase (SMUL_2360; WP_025345454.1) as abundant protein (Keller and Schubert, unpublished data). The gene for this enzyme is surrounded by two transporter genes *SMUL_2359* (Xanthine/uracil/vitamin C family permease; Ghim and Neuhard 1994, Christiansen *et al.* 1997, Liang *et al.* 2001, Schultz *et al.* 2001) and *SMUL_2361* (major facilitator superfamily transporter, Pao *et al.* 1998) in the genome of *S. multivorans* (Goris *et al*. 2014). An involvement of these transporters in the uptake of exogenous benzimidazoles can only be speculated. However, the assumption that imported Bzas are activated by SMUL_2360 was not affirmed since the purified enzyme was unable to utilize DMB as substrate *in vitro* (Keller, data not shown).

The ethanolamine *O*-phosphate (EA-P) linker biosynthesis in *S. multivorans*

Norpseudo-B_{12} of *S. multivorans* and its sister strain *Sulfurospirillum halorespirans* contains a unique EA-P linker moiety connecting the lower ligand and the corrin ring that is replaced by an (*R*)-1-aminopropan-2-ol *O*-2-phosphate (AP-P) linker with an additional methyl group at carbon atom C176 in other natural cobamides (Fig. 1.3, Lenhert 1968, Kräutler *et al.* 2003, Butler *et al.* 2006, Goris *et al.* 2017). L-threonine is the precursor of AP-P in *Salmonella enterica* serovar *Thyphimurium* LT2 (Fig. 1.2). The amino acid is phosphorylated to L-threonine *O*-3-phosphate (L-Thr-P) by the kinase PduX (EC 2.7.1.177) and L-Thr-P is then decarboxylated by the pyridoxal-5'-phosphate (PLP)-dependent L-Thr-P decarboxylase CobD (EC 4.1.1.81) yielding AP-P (Brushaber *et al.* 1998, Fan and Bobik 2008). AP-P is finally attached to the corrin ring compound adenosyl cobyric acid (AdoCby) by the membrane protein adenosylcobinamide phosphate synthase CbiB (EC 6.3.1.10, Zayas *et al.* 2007). The EA-P linker biosynthesis pathway in *S. multivorans* was unraveled in this study. The protein *Sm*CobD (SMUL_1544) was revealed as novel PLP-harboring L-serine *O*-phosphat*e* (L-Ser-P) decarboxylase (2.3, 2.4). This enzyme is the key player in the EA-P linker biosynthesis of *S. multivorans* decarboxylating L-Ser-P to EA-P explaining the lack of a methyl group compared to the L-Thr-P derived AP-P linker. Due to a lack of a gene encoding the L-threonine kinase PduX, which cobamide synthesizers

typically possess (Fan and Bobik 2008, 2009), the precursor for L-Ser-P in *S. multivorans* remains unclear. The absence of an L-threonine kinase that might also phosphorylate L-serine makes the amino acid as L-Ser-P progenitor unlikely. Exogenous applied L-[3-^{13}C]-serine revealed only a minor incorporation of ^{13}C into the EA-P fraction of norpseudo-B_{12} and a predominant one at the methyl group side chains of the corrin ring (2.3, Fig. 6) underlining this hypothesis. Alternatively L-Ser-P could originate from the serine biosynthesis (Pizer 1963). Indeed the amounts of the proteins catalyzing L-Ser-P were significantly higher to those that consume L-Ser-P during L-serine biosynthesis in case of PCE-dependent growth of *S. multivorans* (Goris *et al.* 2014; Goris, data not shown). Hence, an enrichment of L-Ser-P that might serve as EA-P substrate for *Sm*CobD is assumed in *S. multivorans*. In this case a specific L-serine kinase is not required for the norpseudo-B_{12} biosynthesis. The *in vitro* analysis of *Sm*CobD and *Se*CobD revealed that both enzymes are able to decarboxylate L-Thr-P to AP-P and L-Ser-P to EA-P with each a higher activity towards its physiological substrate (see 2.4). At high concentrations of L-Thr-P (1 mM) in the growth medium of *S. multivorans*, the organism predominantly synthesized AP-P containing pseudo-B_{12} (2.3, Fig. 4). This observation revealed that guided cobamide biosynthesis in *S. multivorans* was also able with L-Thr-P and that the CbiB homolog of this bacterium is able to attach EA-P as well as AP-P to the corrin ring, an ability, which was additionally affirmed for *Se*CbiB (Zayas *et al.* 2007). An in depth analysis of CbiB proteins was so far hampered due to difficulties purifying these membrane proteins. The newly gained knowledge that SeCobD is able to decarboxylate L-Ser-P sheds some light on previous experiments. A *S. enterica* Δ*pduX* strain grew slowly under conditions that required the synthesis of coenzyme B_{12} (Fan and Bobik 2008). A potential second low activity threonine kinase complementing the lack in PduX to a small extent was proposed. However, a more feasible explanation is the formation of norcoenzyme B_{12} due to the utilization of L-Ser-P rather than the missing L-Thr-P by *Se*CobD under these conditions. To proof this hypothesis the cobamide fraction of the Δ*pduX* strain could by purified and the linker structure analyzed. The three dimensional structure of the homodimeric *Se*CobD in the presence of PLP, L-ThrP or both yielding the product-aldimine complex was determined long ago (Cheong *et al*. 2002 a, b). According to sequence alignment and preliminary X-ray structure data for *Sm*CobD (2.4, Fig. 1; Seeger and Rayment, data not shown), the overall structure and the active site of the *S. multivorans* decarboxylase seems very similar to *Se*CobD. However, the structure of *Sm*CobD in the presence of PLP, L-Ser-P, or L-Thr-P could not be determined so far. These data

are currently under investigation. Nevertheless one residue important for the L-Ser-P decarboxylation in *Sm*CobD was already revealed by mutagenesis. This enzyme and the homolog of *S. halorespirans* possess the unique Ser242 in their active site that is replaced by a highly conserved threonine residue in L-Thr-P decarboxylases like *Se*CobD. This residue Thr215 in *Se*CobD was shown to bind the phosphate group of PLP in the active site (Cheong *et al.* 2002 a, b). Replacing Ser242 by a threonine (S242T) dropped the L-Ser-P decarboxylation and increased the L-Thr-P decarboxylation rate to similar levels. Unfortunately, exchanging the respective threonine residue (Thr215) of *Se*CobD by a serine reduced the affinity for PLP and hence the decarboxylation rates for both substrates tremendously. This finding proofs a different conformation of *Sm*CobD allowing a serine at this position. Another difference to *Se*CobD is the 28 amino acids longer N-terminus of *Sm*CobD. However, deletion experiments revealed that the first 20 of these residues have no essential function for the activity of the enzyme. The amino acid His26 of *Sm*CobD was shown to be the analog of His8 in *Se*CobD, which is highly important for the binding of L-Thr-P in *Se*CobD (Brushaber *et al.* 1998, Cheong *et al.* 2002 a,b). Most hydrogen bridge bonds of *Se*CobD with L-Thr-P were seen with the phosphate group of this compound (Cheong *et al.* 2002 a,b). Thus, the enzyme was not able to decarboxylate L-threonine *in vitro* (Brushaber *et al.* 1998). The inability of *Sm*CobD to convert L-serine or L-threonine is another evidence for a similar active site and binding patterns to the substrates like in *Se*CobD. A phylogenetic analysis of *Sm*CobD with CobD proteins and aminotransferases of the superfamily of PLP-dependent aspartate aminotransferases displayed a closer relatedness of SmCobD to L-histidinol phosphate aminotransferases (HisC) than to other CobD proteins (see 2.4, Fig. 2). Nevertheless, *Sm*CobD displayed no aminotransferase activity with L-histidinol phosphate *in vitro* (2.4, Fig. S4). These observations might indicate that CobD homologs are derived from aminotransferases and that *Sm*CobD might represent an evolutionary link between HisC and the L-Thr-P decarboxylases.

Guided biosynthesis of diverse cobamides with *S. multivorans*

Norpseudo-B_{12} is the sole cobamide cofactor that is synthesized by *S. multivorans de novo* without the presence of exogenous benzimidazoles or L-Thr-P (Kräutler *et al.* 2003). The uptake of the benzimidazoles DMB, Bza, 5-OHBza, 5-OMeBza, or 5-MeBza triggers the bacterium to produce EA-P containing norcobamides nor-B_{12}, Bza-NCba, 6-OHBza-NCba, 5-

OMeBza-NCba, 5- and 6-MeBza-NCba (see 2.1, 2.2; Tab. 3.1). Exogenous 1 mM L-Thr-P led to the synthesis of pseudo-B_{12} with an AP-P rather than an EA-P linker in the bacterium (see 2.3). A combination of exogenous 1 mM L-Thr-P and 25 µM DMB guided the organism to produce almost exclusively B_{12} with an AP-P linker and DMB as lower ligand (Keller, data not shown). The production of other (Bza)-Cba with exogenous L-Thr-P and diverse benzimidazoles (e.g. Bza, 5-MeBza, 5-OHBza, and 5-OMeBza) may also work in *S. multivorans*. Although L-Thr-P triggers the formation of AP-P cobamides in *S. multivorans*, exogenously applied L-Ser-P did not enhance the EA-P formation indicating either a low efflux, or another fate of this compound in the bacterium (see 2.3). A summary of the different cobamides *S. multivorans* can synthesize is shown in Figure 3.2.

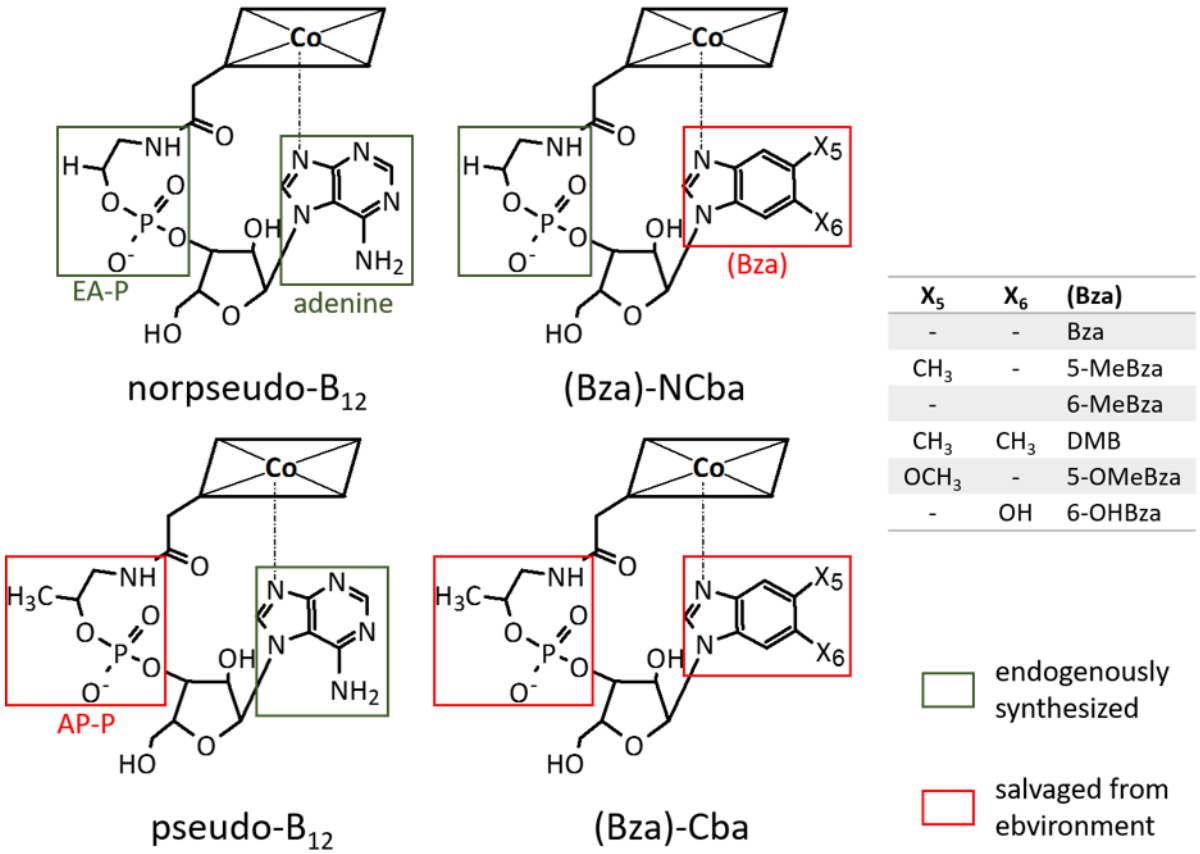


Figure 3.2: Diversity of cobamide cofactors that can be produced in *S. multivorans*. The frames mark the linker and lower bases that are either endogenously synthesized (*green*), or derived from environmentally affiliated precursors (*red*).

S. multivorans is the only known B_{12} *de novo* synthesizing prokaryotic species applicable for the synthesis of not just a set of AP-P cobamides with varying lower ligands, but also different norcobamides. Unfortunately with an approximate amount of 0.5 mg/l cobamide purifieable from late exponential *S. multivorans* cells on pyruvate/fumarate medium this organism produces less B_{12} than biotechnologically relevant strains (Schubert 2017, Bykhovsky *et al.* 1998, Martens *et al.* 2002). Typical industrial applied strains like *Pseudomonas denitrificans* or *Propionibacterium shermanii* were engineered to produce 60 – 200 mg/l vitamin B_{12}. An enhancement of the cobamide quantities by genetic engineering of *S. multivorans* would be highly challenging, since no protocol for systematic genetic modification is available for this bacterium by now. Even though *S. multivorans* does not synthesize biotechnological cobamide quantities, it can be easily used to produce cobamide standards for low scale applications like HPLC or for salvaging experiments as medium complementations.

The effect of the guided biosynthesis products on the PCE metabolism of *S. multivorans*

The catabolic tetrachloroethene reductive dehalogenase PceA of *S. multivorans* was previously shown to incorporate the unusual cobamide norpseudo-B_{12} and offered a significantly higher PCE dechlorination rate than other PceA homologs containing purinyl- or DMB-Cbas (Kräutler *et al.* 2003, Yan *et al.* 2012, Schubert and Diekert 2016; Schubert, unpublished data). Norpseudo-B_{12} is bound in *Sm*PceA in a special and novel curled 'base-off' conformation (Bommer *et al.* 2014). In this conformation the adenine of norpseudo-B_{12} is solvent accessible and showed some hydrogen bonding with the protein backbone due to its hydrophilic character. Other hydrogen bridges were observed between the carboxamide side chain of pyrrole ring C, the amino part of the EA-P linker, and the 5-hydroxy group of the ribose moiety of the Cba. The carbon atom C176 of the EA-P linker is very close to a β-sheet of PceA and the incorporation of the methyl group of AP-P was assumed to require conformational changes of the whole protein. The binding of PCE and the enzyme catalysis of PceA was shown at the upper face of the corrin ring like in the other known cobamide-dependent enzymes, which excluded a potential involvement of the adenine base that is far away from the cobalt center and thus mainly responsible for binding of the cofactor to the protein (Gruber *et al.* 2011, Bommer *et al.* 2014, Bridwell-Rabb and Drennan 2017). One major question of this study was, if norpseudo-B_{12} with its specific and unique structure was essential for the PceA activity.

This assumption was proven wrong since several benzimidazole-containing norcobamides that were synthesized in *S. multivorans* by guided biosynthesis could efficiently replace norpseudo-B_{12} without any negative effect for the bacterium (see 2.2, Tab. 3.1). These NCbas are Bza-, 5-OMeBza-, and 6-OHBza-NCba. Crystallization and X-ray analysis of PceA containing 6-OHBza-NCba or 5-OMeBza-NCba revealed that the benzimidazoles adopted almost the same position like adenine of norpseudo-B_{12} in the active site with the same curled 'base-off' state of the nucleotide loop. A change in the accommodation of the water molecules in the solvent accessible binding pocket around the benzimidazole bases allowed them to locate at the same position like adenine. Since most known protein structures were gained from cobamide-dependent enzymes containing DMB-Cba, PceA of *S. multivorans* belongs to the first structural investigated enzymes bearing an adenine-Cba (Mancia *et al.* 1996, Reitzer *et al.* 1999, Payne *et al.* 2015). Moreover, due to the structures with 5-OMeBza-NCba and 6-OHBza-NCba it is the first enzyme, in which the incorporation of purinyl- and benzimidazolyl-cobamides was analyzed. The space in front of C5 and C6 of the benzimidazoles in PceA was not closely packed indicating enough space for methyl groups instead of hydroxyl or methoxy groups as substituents at both carbon atoms (2.2, Fig. 7). Interestingly, the activity of a PceA fraction that was saturated with 5- and 6-MeBza-NCba in approximately equimolar amounts was reduced to 50 % compared to norpseudo-B_{12} containing PceA (2.2, Fig. 6; Tab. 3.1). Additionally, this synthesis of the MeBza-NCbas in *S. multivorans* had a negative effect of the maturation rate of cytoplasmic prePceA to periplasmic PceA (2.2, Fig. 5B; John *et al.* 2006, Palmer and Berks 2012). Due to an impossibility of resolving the structure of PceA containing the MeBza-NCbas it is actually not possible to predict, if the incorporation of both or either 5-MeBza-, or 6-MeBza-NCba into prePceA lead to the negative effects. An optimization of the PceA purification protocol might lead to a separation of PceA load with 5-MeBza- and 6-MeBza-NCba, which could then separately be investigated towards their PCE dechlorination activity. The three dimensional structures of PceA with exclusive 5-MeBza-, or 6-MeBza-NCba could moreover unfold possible conformational changes preventing an efficient PCE-dechlorination. However, there was no significant negative effect observed for the PCE-dependent growth of *S. multivorans* with exogenous 5-MeBza indicating an excess of expressed *pceA* than required for the PCE metabolism (2.2, Fig. 5A). Strong negative effects on growth and PceA dechlorination rate were observed for DMB containing nor-B_{12} (2.1, 2.2; Tab. 3.1). A more than 90 % reduced activity, a highly reduced amount of the periplasmic holo

enzyme PceA and the detection that less than 2 % of the purified PceA molecules contained a cobamide strongly suggested a very poor uptake of nor-B_{12} into the RDase. Since the corrin ring of cobamides is to big to fit through the substrate- or solvent channel of PceA, it is assumed that the cobamide binds to prePceA prior to the correct folding of the protein wrapping around the B_{12} (Bommer *et al.* 2014). As predicted from the PceA structure containing 6-OHBza-NCba or 5-OMeBza-NCba that showed no predictable clash of the 5- and 6-methyl group of DMB with the protein, this incompatibility of PceA for nor-B_{12} remains unclear. The structural investigated enzymes that contain a DMB-cobamide in 'base-off' conformation including *Np*RdhA of *Nitratireductor pacificus* pht-3B were shown to bind DMB in a hydrophobic pocket (Mancia *et al.* 1996, Reitzer *et al.* 1999, Payne *et al.* 2015). However, while the lower base of the cobamide in *Np*RdhA was protected from the solvent, it is solvent accessible in *S. multivorans* creating a hydrophilic environment (Bommer *et al.* 2014). Difficulties of the incorporation of NCbas bearing benzimidazole bases with hydrophobic methyl groups at C5 or C6 (MeBza and DMB) into this hydrophilic binding pocket might cause the observed negative effects on PceA maturation and activity. Another possible explanation of the inefficient incorporation of nor-B_{12} into PceA lies in the high tension of the DMB-cobamides for the cobalt-coordinating 'base-on' conformation (Fieber *et al.* 2003). An experiment with pseudocoenzyme B_{12} and coenzyme B_{12} revealed that the adenine-Cba was mainly present in the 'base-off' form and the DMB-Cba was predominantly present in the 'base-on' form in aqueous solutions. Since the cobamides in PceA are bound in the curled 'base-off' state and the EPR data displayed no involvement of a 'base-on' Co^{3+} state during catalysis, the cofactor is hypothized to require the 'base-off' conformation before it can be incorporated into PceA (Bommer *et al.* 2014). The very low incorporation rate of nor-B_{12} into PceA might be caused by a low amount of accessible 'base-off' cobamide after biosynthesis. Some benzimidazole-harboring NCbas efficiently replaced norpseudo-B_{12} in PceA without any negative effect on the enzyme activity. Another question was, if PceA relies on norcobamide cofactors or if it's able to incorporate AP-P cobamides as well. The synthesis of AP-P containing pseudo-B_{12} with exogenous L-Thr-P was revealed for *S. multivorans* (see 2.3). As previously assumed the structure of PceA suggested not enough space for the additional methyl group of pseudo-B_{12} due to a β sheet near to C176 of the cobamide (Bommer *et al.* 2014). Indeed, the pseudo-B_{12} biosynthesis in *S. multivorans* revealed some negative effects on the PCE metabolism (see 2.3). A delayed growth behavior on PCE-medium as well as a reduced amount

and a coherent reduction in the PCE dechlorination activity of PceA in cell free extracts of the bacterium were observed. The cobamide extraction of purified PceA from mainly pseudo-B_{12} producing *S. multivorans* unraveled that norpseudo-B_{12} was preferentially incorporated into PceA (Treder 2015, unpublished data). Moreover, the amount of PceA was reduced under these conditions as indicated by immuno-blotting (2.3, Fig. 5B). PceA was shown to prefer norcobamides and low incorporation rate of pseudo-B_{12} supports the assumption of an incompatibility of PceA for an AP-P Cba due to a clash of the methyl group at C176 with the protein (Bommer *et al.* 2014). This proximity of a β-sheet of PceA to C176 of the cobamide cofactor seemingly hamper the incorporation of pseudo-B_{12} explaining the native biosynthesis of the unique EA-P containing norpseudo-B_{12} in this bacterium. Unfortunately, the determination of the PceA structure with pseudo-B_{12} is hindered by the inhomogeneity of the purified protein fraction caused by the predominant incorporation of norpseudo-B_{12}. The compatibility of the cobamide-dependent enzymes of AP-P Cba producing prokaryotes is affirmed by the high abundance of these Cbas. The compatibility of such enzymes for norcobamides was not studied extensively so far. Preliminary data with *S. enterica* showed that norpseudo-B_{12} and nor-B_{12} as sole cobamides efficiently replaced the AP-P Cba in the organism (see 2.3, Keller and Schubert, data not shown; Zayas *et al*. 2007, Anderson and Roth 2008). Some cobamides synthesized by *S. multivorans* were shown to negatively influence the PCE metabolism of the OHRB. This observation implies the lack of a selective mechanism preventing *S. multivorans* to produce cobamides incompatible to PceA. Negative effects on the Cba-dependent growth caused by the synthesis of DMB-Cba were previously observed for *Sporomusa ovata* that naturally produces phenolyl-Cbas (Stupperich and Eisinger 1989, Stupperich *et al.* 1989, Mok and Taga 2013). However, *Lactobacillus reuteri*, and *Sinorhizobium meliloti* incorporated only a subset of exogenous bases into cobamides and some bacteria like *Dehalococcoides mccartyi* and *Rhodobacter sphaeroides* contain a cobinamide amidohydrolase CbiZ (EC 3.5.1.90) to remove the nucleotide loop of cobamides with incompatible lower bases and rebuilt it with the correct base (Woodson and Escalante-Semerena 2004, Grey and Escalante-Semerena 2009, Yi *et al.* 2012, Crofts *et al.* 2013). The lack of such a protective mechanism in *S. multivorans* indicates a compatibility of PceA with the respectively produced cobamides at natural concentrations of DMB or 5-MeBza. Indeed, no significant effect on the PCE-metabolism of *S. multivorans* was observed at 100 nM exogenous DMB (see 2.1). The environmental concentration of DMB in activated sludge, the

bacterium was isolated from, is definitely much lower (Scholz-Muramatsu *et al.* 1995). Anaerobic TCE-dechlorinating enrichment cultures from contaminated groundwater or soil contained maximal solvent concentrations of 5 nM DMB (Men *et al*. 2015). Creek water contained less than 10 pM DMB and creek bank soil and eucalyptus grove soil less than 1 pmol/g DMB (*Crofts et al.* 2014b). Hence, natural concentrations of DMB are not causing negative effects in *S. multivorans* due to a negligible production of nor-B_{12}. *S. multivorans* PceA displayed a tremendously higher PCE dechlorination activity than the PceA homologs of other OHRB (Schubert and Diekert 2016). The assumption that norpseudo-B_{12} with its unique EA-P linker and adenine ligand enhances the enzyme activity was not confirmed in this study since also Bza, 6-OHBza, or 5-OMeBza-NCba-harboring PceA dechlorinated PCE with a similar activity. These finding showed that cobamides efficiently binding PceA assured a rapid dechlorination of PCE. Cobamides are able to dechlorinate organohalogens not only in an enzyme-bound state, but also in an abiotic form. For this ability the cobamides require the Co^{1+} 'base-off' state, which is reached at a highly negative redox potential less than -450 mV. The applied spectrophotometrical assay revealed that norpseudovitamin B_{12} dechlorinated trichloroacetate much faster than pseudovitamin B_{12}, norvitamin B_{12}, or vitamin B_{12} (2.1, Fig. 7; Neumann *et al*. 2002). These observations unraveled a positive effect of the EA-P linker and the adenine ligand on the dechlorination rate. Subsequent redox analysis revealed that more than 90 % of the norpseudovitamin B_{12} molecules were reduced to the Co^{1+} 'base-off' conformation in the assay, whereas only 50 % pseudovitamin B_{12} and less than 10 % vitamin or norvitamin B_{12} were reduced to the active state (Keller, unpublished data). These data affirm that the EA-P linker and the adenine base increase the potential of the cobamide required for the reduction to the active Co^{1+} state in the abiotic dechlorination assay. Titanium (III) citrate was not able to reduce the cobamides completely, but if a reducing agent was able to achieve their complete reduction all cobamides might abiotically dechlorinate trichloroacetate with the same conversion rate. However, the potential required to reduce Co^{2+} norpseudo-B_{12} to the active Co^{1+} form in PceA is with -0.38 V more positive (Kräutler *et al*. 2003). Since norcobamides with certain benzimidazole bases replaced norpseudo-B_{12} efficiently in PceA the high activity of this enzyme was revealed to not be caused by the unique structure of the *S. multivorans* native cobamide cofactor (2.2). This project gained new insights into the biosynthesis and diversity of cobamides and their utilization in cobamide-dependent enzymes. The unique parts of the norpseudo-B_{12} biosynthesis were unraveled in this project,

especially the EA-P linker formation. The RDase PceA of *S. multivorans* was revealed to preferably incorporate norcobamides with a subset of purine or benzimidazole bases. However, other investigated RDases display a more restricted or broader cobamide requirement and a general indication for RDases is hence not possible (Reinhold *et al.* 2012, Yi *et al.* 2012, Yan *et al.* 2013, Payne *et al.* 2015). PceA is the first cobamide-dependent enzyme that was structurally investigated harboring either a purinyl, or a benzimidazolyl cobamide. The characterization of the cobamide compatibility and incorporation of other RDases and in general cobamide-dependent enzymes is a nice outlook to extend this knowledge about the reaction mechanisms of these enyzmes and the functions of the cobamide cofactors and especially their nucleotide loops.

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List of publications Sebastian Keller

Published manuscripts

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Keller S., Ruetz M., Kunze C., Kräutler B., Diekert G., Schubert T. (2014) Exogenous 5,6-dimethylbenzimidazole caused production of a non-functional tetrachloroethene reductive dehalogenase in *Sulfurospirillum multivorans*. Environ Microbiol. 2014 Nov; 16(11):3361-9. doi: 10.1111/1462-2920.12268. Epub 2013 Oct 6.

Non-published manuscripts

Keller S., Schubert T. Functional analysis of a unique L-serine *O*-phosphate decarboxylase essential for the norcobamide biosynthesis in *Sulfurospirillum multivorans*.

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Keller S., Cindy Kunze, Martin Bommer, Christian Paetz, Riya C. Menezes, Aleš Svatoš, Holger Dobbek, Torsten Schubert. Molecular inside view of how benzimidazoles steer cobamide cofactor biosynthesis and utilization in the organohalide-respiring bacterium *Sulfurospirillum multivorans*.

Status: 07/28/2017 submitted to Molecular Microbiology and rejected (28/08/2017)

prepared for publication in Journal of Bacteriology (planned submission: Oct 2017)

Declaration of honor

I hereby certify that I, Sebastian Keller, am familiar with the relevant course of examination for doctoral candidates of the faculty of Biology and Pharmacy at the Friedrich Schiller University Jena. This thesis has been composed by me and is based on my own work, unless stated otherwise. I did not use any sections of text from a third party or from personal dissertations without identifying them as such. All assistance, personal communication, and sources are acknowledged in this thesis. I did not enlisted the assistance of a doctoral consultant and no third parties have received any monetary benefits from the work connected to the submitted dissertation. I have not already submitted the dissertation as an examination paper for a state of other scientific examination. Moreover, I certify that I have not submitted the same, a substantially similar, or a different paper to another postsecondary school.

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